UNIVERSIDADE DO ALGARVE

FACULDADE DE CIÊNCIAS DO MAR E DO AMBIENTE

POLYCYCLIC AROMATIC HYDROCARBONS AND OXIDATIVE STRESS MARKERS IN THE CLAM *Ruditapes decussatus* FROM THE RIA FORMOSA (PORTUGAL)

(Tese definitiva para a obtenção do grau de doutor no ramo de Ciências e Tecnologias do Ambiente, especialidade de Ambiente Aquático)

LUÍSA PAULA VIOLA AFONSO BARREIRA

Orientador: Doutora Maria João da Anunciação Franco Bebianno

Constituição do Júri:

Presidente: Doutor João Pinto Guerreiro

Vogais: Doutora Michèle Roméo

Doutora Maria Ana Dias Monteiro Santos

Doutora Maria João da Anunciação Franco Bebianno

Doutora Ana Maria de Lima Viegas Gonçalves Crespo

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NOME: Luísa Paula Viola Afonso Barreira

FACULDADE: Faculdade de Ciências do Mar e do Ambiente

ORIENTADOR: Maria João Bebianno

DATA: 10/04/2007

TÍTULO DA TESE: Polycyclic Aromatic Hydrocarbons and Oxidative Stress Markers in the clam *Ruditapes decussatus* from the Ria Formosa (Portugal)

RESUMO

Este trabalho envolveu o estudo de hidrocarbonetos aromáticos policíclicos (PAHs) na amêijoa *Ruditapes decussatus* da Ria Formosa, bem como a sua relação com marcadores de stress oxidativo. A variação espacial e sazonal da concentração e possíveis fontes de PAHs foi avaliada nos sedimentos superficiais e na parte edível da amêijoa, tendo as variações sazonais sido as mais importantes. A actividade de enzimas antioxidantes e a peroxidação lipídica (LPO) na glândula digestiva da amêijoa foram também mais afectadas por factores sazonais do que espaciais. A Superóxido Dismutase (SOD) e a Catalase (CAT) não apresentaram qualquer relação com a concentração de PAHs, parecendo a CAT estar particularmente relacionada com o ciclo reprodutivo da amêijoa. Por outro lado, as Glutationo Peroxidases (GPx) e LPO apresentaram uma relação inversa com a concentração destes compostos, o que pode estar relacionado com um estado precário dos organismos associado a toxicidade dos PAHs, embora não se tenham observado danos oxidativos.

De modo a clarificar a relação entre os PAHs e os parâmetros de stress oxidativo procedeu-se ao transplante de amêijoas entre diferentes locais, tendo-se observado uma acumulação significativa de PAHs nestes organismos. As actividades da Benzo[*a*]pireno Hidroxilase (BPH) e Glutationo S-Transferase (GST) na glândula digestiva aumentaram em resposta ao aumento da concentração de PAHs nos tecidos da amêijoa confirmando a metabolização de PAHs neste tecido. A exposição e o metabolismo de PAHs levaram também à indução de stress oxidativo com o consequente aumento na actividade de SOD citosólica e T GPx nas brânquias e da actividade da SOD mitocôndrial e da CAT na glândula digestiva juntamente com um aumento de peroxidação lipídica.

Assim, conclui-se que as enzimas antioxidantes não são bons biomarcadores de exposição a PAHs em *R. decussatus*, enquanto que a BPH, a GST e LPO revelam algum potencial devendo no entanto ser estudada a sua variação sazonal.

Palavras-chave: Hidrocarbonetos aromáticos policíclicos; enzimas antioxidantes; biomarcadores; *Ruditapes decussatus*; Ria Formosa; transplante.

Polycyclic Aromatic Hydrocarbons and Oxidative Stress Markers in the clam *Ruditapes decussatus* from the Ria Formosa (Portugal)

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) were studied in clams *Ruditapes decussatus* from the Ria Formosa lagoon as well as their relationship with oxidative stress markers. Both the seasonal and spatial variations of PAHs levels and their sources were assessed in surface sediments and clam whole soft tissues. PAHs concentrations and sources were seasonal rather than spatial dependent in the two compartments as well as antioxidant enzymes activities and lipid peroxidation levels (LPO) in the clam digestive glands. Superoxide dismutase (SOD) and Catalase (CAT) were not related to the PAHs concentrations, instead, CAT was probably more related to the clam reproductive cycle. Glutathione peroxidases (GPxs) and LPO were negatively related to PAHs concentrations which may indicate a precarious state of the exposed clams associated with PAHs toxicity although not causing membrane oxidative damage. A transplant experiment was planned to clarify PAHs metabolism and effects on these parameters. Significant PAHs accumulation was observed accompanied by inductions of Benzo[*a*]pyrene Hydroxylase (BPH) and Glutathione S-Transferase (GST) in the digestive gland of clams, indicating PAHs metabolisation by clams. PAHs exposure and metabolisation induced also oxidative stress in both the gills and digestive gland which caused increases in Cyt SOD and T GPx activities in the gills and Mit SOD and CAT activities and in LPO products in the digestive gland.

Therefore, antioxidant enzymes were not considered effective biomarkers of PAHs exposure in *R. decussatus* while BPH, GST and LPO reveal some potential although their seasonal variation should be studied in this species.

Keywords: Polycyclic aromatic hydrocarbons; antioxidant enzymes; *Ruditapes decussatus*; biomarkers; Ria Formosa; transplantation.

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ABBREVIATIONS AND ACRONYMS

4- HNE	4-hydroxyalkenals
A	Anthracene
Ac	Acenaphthene
Ace	Acenaphthylene
AChE	Acetyl Cholinesterase
AsPx	Ascorbate peroxidase
BaA	Benzo[*a*]anthracene
BAF	Bioaccumulation Factor
BaP	Benzo[*a*]pyrene
BbF	Benzo[*b*]fluoranthene
BCF	Bioconcentration Factor
BkF	Benzo[*k*]fluoranthene
BPer	Benzo[*g,h,i*]perylene
BPH	Benzo[*a*]pyrene Hydroxylase
BSA	Bovine serum albumin
BSAF	Biota-sediment Accumulation Factor
CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
Ch	Chrysene
CI	Condition Index
Cu/Zn SOD	Copper and zinc containing superoxide dismutase
Cyt P450	Cytochrome P450
Cyt SOD	Cytosolic SOD
DBA	Dibenzo[*a,h*]anthracene
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane
DG	Digestive Gland
DNA	Deoxyribonucleic acid
DTD	DT-diaphorase
EC	Enzyme Classification number

EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
Eq	Equivalent
F	Fluorene
FAD	Flavin Adenine Dinucleotide
Fe-SOD	Iron containing superoxide dismutase
FID	Flame Ionization Detector
Fluor	Fluoranthene
G	Gills
GC	Gas Chromatography
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Reduced glutathione
GSSG	Glutathione disulphide
GST	Glutathione S-transferase
H	Hexane
HPLC	High Performance Liquid Chromatography
IP	Indene[123-*cd*]pyrene
KDa	Kilodaltons
K_{ow}	Octanol/water coefficient
Log	Logarithm
LPO	Lipid peroxidation
MDA	Malondialdehyde
Mit SOD	Mitochondrial SOD
MFO	Mixed Function Oxygenase System
Mn-SOD	Manganese containing superoxide dismutase
MT	Metallothionein
N	Naphthalene
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
OC	Organic Carbon
OM	Organic Matter
P	Phenanthrene

PAH	Polycyclic Aromatic Hydrocarbons
PC	Principal Component
PCA	Principal Components Analyses
PCB	Polychlorinated Biphenyls
PLS	Partial Least Squares
PUFA	Polynunsaturated Fatty Acids
Py	Pyrene
ROS	Reactive Oxygen Species
SD	Standard Deviation
Se-GPx	Selenium dependent Glutathione Peroxidase
SOD	Superoxide Dismutase
TBT	Tributyltin
T GPx	Glutathione Peroxidase Total fraction
tPAH	Total Polycyclic Aromatic Hydrocarbons
Tris	Tris (hydroxymethyl) aminomethane
UDP	Glucoronyl Transferase
UV	Ultra Violet radiation

1. GENERAL INTRODUCTION

POLYCYCLIC AROMATIC HYDROCARBONS AND OXIDATIVE STRESS MARKERS IN THE CLAM *Ruditapes decussatus* FROM THE RIA FORMOSA (PORTUGAL)

1.1. POLYCYCLIC AROMATIC HYDROCARBONS

1.1.1. Physico/chemical properties

Polycyclic Aromatic Hydrocarbons (PAHs) constitute a class of organic compounds composed of two or more fused aromatic rings. Naphthalene ($C_{10}H_8$), with two fused aromatic rings, is the lowest molecular weight PAH. PAHs with nine or more aromatic rings have been identified in the resin-asphaltene fractions of petroleum (Neff, 2002). PAH molecules have a flat structure without substituent groups although some derivatives containing nitrogen, sulphur and oxygen heteroatoms may be included in this class of contaminants.

PAHs present generally high melting and boiling points, low vapour pressure and very low water solubility. They are very lipophilic, a characteristic measured by their high octanol/water partition coefficient, and chemically rather inert. However, these characteristics vary according to their number of rings and molecular weight. Table 1.1 shows the structure and physico/chemical characteristics of some PAHs usually found in the environment.

1.1.2. Formation and sources

PAHs may be formed by a variety of mechanisms (Neff, 2002):

- incomplete combustion or pyrolysis at high temperature (e.g. 700°C) of organic materials (pyrogenic PAHs), which is a very fast mechanism;
- rearrangement and transformation of biogenic organic materials at moderate temperatures of 100-300°C to form fossil fuels (petrogenic PAHs), which is a very slow process (e.g., millions of years);

– transformation of certain organic compounds in soils and sediments (diagenic PAHs), which is a relatively rapid process (days to years);

– direct biosynthesis by organisms (biogenic PAHs).

From these, combustion of organic matter, including fossil fuels, is thought to be the major source of PAHs to the environment (Laflamme & Hites, 1978; Wakeham *et al.*, 1980; Gschwend & Hites, 1981; Simcik *et al.*, 1999; Slater *et al.*, 2002). It generates a wide variety of PAHs ranging from naphthalene to complex PAH polymers, particularly if combustion takes place in an oxygen-deficient environment (fuel-rich combustion mixture). During combustion in an oxygen-deficient atmosphere, organic matter is oxidized to low molecular weight organic molecules that condense as the combustion mixture cools to form complex new molecular structures, including aromatic and polycyclic aromatic hydrocarbons, a process called pyrolysis/pyrosynthesis (Neff, 2002).

Table 1.1 – Physico/chemical properties of PAHs.

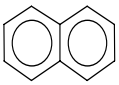
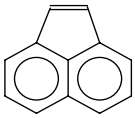
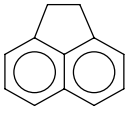
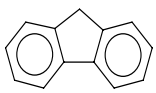
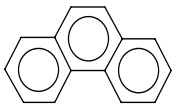
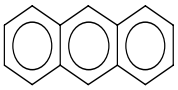
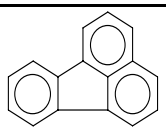
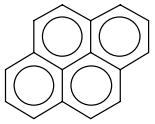
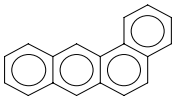
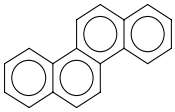
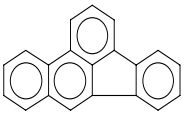
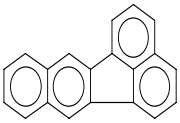
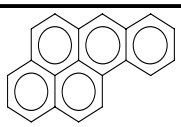
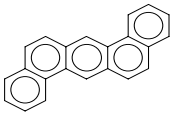
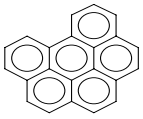
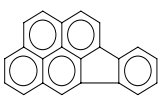
Name	Structure	Molecular Weight	Solubility (mmol l^{-1})	Log K_{ow}
Naphthalene*		128.2	2.4×10^{-1}	3.37
Acenaphthylene*		152.2	1.1×10^{-1}	4
Acenaphthene*		154.2	2.5×10^{-2}	3.92
Fluorene*		166.2	1.1×10^{-2}	4.18

Table 1.1 (Cont.)

Name	Structure	Molecular Weight	Solubility (mmol l^{-1})	Log K_{ow}
Phenanthrene*		178.2	2.6×10^{-2}	3.24
Anthracene*		178.2	2.5×10^{-4}	4.54
Fluoranthene*		202.3	1.3×10^{-3}	5.22
Pyrene*		202.3	6.5×10^{-4}	5.18
Benzo[*a*]anthracene*		228.3	4.8×10^{-5}	5.91
Chrysene**		228.3	1.3×10^{-5}	5.84
Benzo[*b*]fluoranthene*		252.3	6×10^{-6}	5.8
Benzo[*k*]fluoranthene*		252.3	3.2×10^{-6}	6
Benzo[*a*]pyrene*		252.3	1.5×10^{-5}	6.04
Dibenzo[*a,h*]anthracene**		278.4	1.8×10^{-5}	7.11
Benzo[*g,h,i*]perylene*		276.3	9.7×10^{-7}	6.05
Indene[123-*cd*]pyrene**		276.3	6.9×10^{-7}	7.04

* Dabestani & Ivanov (1999); ** Baumard *et al.* (1998a)

Pyrogenic PAH sources include: exhaust gases and ashes produced in organic matter combustion (Simcik *et al.*, 1999; Oda *et al.*, 2001, Slater *et al.*, 2002; Kristensson *et al.*, 2004); soot particles (Prahl & Carpen, 1983; Mitra *et al.*, 1999; Fernandes & Brooks, 2003; Vardar *et al.*, 2004); waste incineration (Johansson & von Bavel, 2003; Sul *et al.*, 2003); coke and asphalt production (Margarido, 1999); aluminium production (Jongeneelen, 2001); heat and electricity generation in power plant facilities and in domestic furnaces (Kjällstrand & Olsson, 2004; Kakareka *et al.*, 2005); forest fires (Bouloubassi & Saliot, 1993; Gabos *et al.*, 2001); tobacco smoke (Baek & Jenkins, 2004); coal tar, creosote and related wood preservatives (Hyötyläinen & Olkari, 1999; Zhurinsh *et al.*, 2005), among others.

PAHs resulting from incomplete combustions may be transported as gases or aerosols or adsorbed to atmospheric particles (Manoli & Samara, 1999). Airborne PAHs are relatively short-lived, in the order of a few tens of hours (Golomb *et al.*, 2001). Thus in dry air, PAHs will not travel very far from the emission sources, and usually are deposited a few to tens of kilometres away from the emission source by a process which is known as dry deposition. Dry deposition includes also the direct impact on land or water of particulate PAHs (Manoli & Samara, 1999). The total amount of PAH atmospheric inputs through dry deposition in Chesapeake Bay was about 4400 Kg per year (Arzayus *et al.*, 2001). Atmospheric fallout includes also wet deposition of particles and vapours. Wet deposition enters the water surface by precipitating hydrometeors (raindrops and snow flakes). Rainwater has on numerous occasions been shown to contain many organic compounds including PAHs, in concentrations sometimes much higher than in the receiving water body (Ollivon *et al.*, 1999; Polkowska *et al.*, 2000; Grynkiewicz *et al.*, 2002). Wet deposition of PAHs may also originate from regional urban/industrial sources. PAH inputs to the New England coastal areas, for example, determined by Golomb (2001) were much higher by wet deposition (720 – 831 ng/m^{2}cm of precipitation) than by dry deposition (9.3 – 95 ng/m^{2}h).

Coal and petroleum are rich sources of petrogenic PAHs (Neff, 2002). Most of the PAHs in coal are tightly bound in the coal structure and cannot be easily leached out. However, coal dust and particles may contribute to the total extractable PAHs in sediments of bays and estuaries (French, 1998; Walker *et al.*, 2005). Crude usually contains much higher concentrations of extractable PAHs than coals do (Page *et al.*, 1998). However, the PAH composition of coal and crude oil is usually similar (Neff, 2002). Petrogenic PAHs enter the marine environment from natural oil seeps, erosion of coal, peat, and oil shale deposits, oil and coal spills, discharges of treated and untreated ballast and bilge water from oil tankers and other ships, and effluents from oil refineries, oil/water separations on production platforms, coal-fired power plants, and municipal sewage treatment plants (Blumer & Sass, 1972; Bouloubassi & Saliot, 1993; Ali *et al.*, 1995; Lee & Page, 1997; Porte *et al.*, 2000; Medeiros *et al.*, 2005).

Municipal wastewaters are a relevant PAH source to surface waters. PAH concentrations in raw municipal wastewaters may vary considerably, depending on the amount of industrial effluents possibly co-treated with domestic wastewaters (Harrison *et al.*, 1975). Treated wastewaters generally contain much lower concentrations (<1 µg/l) due to their removal by adsorption to particles, biodegradation or volatilisation (Richards & Shieh, 1986; Marttinen *et al.*, 2003). A significant amount of PAHs carried to surface waters by sewers derives also from urban runoff (Manoli & Samara, 1999; Murakami *et al.*, 2005). Urban runoff consists of the storm water from impervious areas, such as roads, motorways, paved parking lots, roofs, sidewalks, etc. As a consequence, urban runoff contains PAHs deposited on these surfaces, as well as mobile related PAHs from gasoline and oil drips or spills, exhaust products, tyre particles, and bitumen from road surfaces (Maltby *et al.*, 1995; Manoli & Samara, 1999; Dickhut *et al.*, 2000; Menzie *et al.*, 2002). The relative contribution of urban-runoff to receiving waters is site-specific, dependent on the relative magnitudes of the wet and dry weather discharges (Manoli & Samara, 1999).

Although specific sources are known to be responsible for the introduction of PAHs in surface waters, their occurrence in the environment cannot always be related to just one particular source. Instead, the PAHs found in the environment are, generally, the result of a mixture of different PAHs from a large variety of origins. However, each PAHs source usually originates a specific molecular pattern of individual parent or modified (alkylated) aromatic hydrocarbons (Lake *et al.*, 1979; Baumard *et al.*, 1999a; Page *et al.*, 1999; Savinov *et al.*, 2000; Guinan *et al.*, 2001). In this way, PAH sources identification generally relies on chemical analyses, which generate hydrocarbon "fingerprints" based on the individual hydrocarbon analytes that are diagnostic of the source (Boehm *et al.*, 1997). Petrogenic PAHs are characterized by homologous families of related PAHs (naphthalenes, fluorenes, phenanthrenes, dibenzothiophenes and chrysenes), where the unsubstituted parent PAHs for each family are more abundant than the alkylated homologues; biogenic PAHs generated by biologic processes or by the early stages of diagenesis in marine sediments are generally enriched in perylene (a 4-ring unsubstituted PAHs); and pyrogenic PAHs, are dominated by the parent compounds of the 3-, 4- and 5-ring PAHs (Page *et al.*, 1999). In this way, differences in ratios of parent to alkyl substituted PAH congeners can be used to distinguish between petrogenic and various types of pyrogenic PAH assemblages in environmental samples (Bence *et al.*, 1996; Zeng & Cherrir, 1997)

Ratios between some individual parent PAHs may also be used to identify PAHs originating processes (Wang *et al.*, 1999; Woodhead *et al.*, 1999). For example, phenanthrene (P) is thermodynamically more stable than anthracene (A), so high P/A ratios are observed in petrogenic pollution and low (<10) in pyrolytic pollution (Soclo *et al.*, 2000). Also, fluoranthene (Fluo) and pyrene (Py) are often associated and considered typical pyrogenic products generated from high temperature condensation of low molecular weight compounds. During the combustion process, however, pyrene is more stable than fluoranthene hence pyrolytic products

are usually characterized by a predominance of fluoranthene over pyrene (Fluo/Py ratio >1) (Baumard *et al.*, 1999a). These ratios, however, are not conclusive in the analysis of petrogenic and pyrogenic origin and the analysis of alkylated PAHs are generally required to further substantiate their use.

1.1.3. Polycyclic Aromatic Hydrocarbons in seawater

After entering the aquatic environment, the behaviour and fate of PAHs depend on their physicochemical properties (Witt, 1995; Dabestani & Ivanov, 1999; Kirso *et al.*, 2001). Volatilisation, dissolution, photodegradation, adsorption onto suspended solids and subsequent sedimentation, biotic and abiotic degradation, uptake by aquatic organisms and accumulation, are all major processes to which water PAHs are subjected (Witt, 2002). PAHs solubility is very low and decreases with increasing molecular weight (see Table 1.1), thus, concentration of dissolved PAHs in water is very low. Conversely, PAHs associate easily with particulate matter and are finally deposited in the sediments (Witt, 2002).

Table 1.2 shows the concentration of dissolved PAHs in marine waters worldwide. PAH concentrations vary widely, being usually low or undetectable at offshore sites (Baltic Sea, Eastern Mediterranean, for example) or very high in many coastal and estuary sites (Bouloubassi & Saliot, 1993; Burt & Ebell, 1995; Gustafson & Dickhut, 1997; Law *et al.*, 1997).

Due to their lipophilic behaviour, PAHs have a natural tendency to adsorb to particles. The sorption process can be represented as an equilibrium between the aqueous phase and the non-aqueous (e.g. octanol) as described by a partition coefficient, K_{ow} (Kirso *et al.*, 2001; Tremblay *et al.*, 2005). Likewise, the degree to which a particular PAH will sorb to a particular sorbant is strongly influenced by the organic carbon content of the sorbant (Borglin *et al.*, 1996) and also by temperature and salinity (Tremblay *et al.*, 2005).

Dissolved organic matter (DOM) has been postulated to increase the solubility of PAHs in seawater (Haitzer *et al.*, 1999; Akkanen & Kukkonen, 2001). Colloids are considered as a part of DOM, and show a great capacity to sorb hydrophobic contaminants in general, and PAHs in particular (Wijayaratne & Means, 1984). Temperature has a small influence, increasing PAHs solubility by a factor of two to five depending on the specific PAH (Whitehouse, 1984). Salinity, however, has the opposite effect. The effect of salinity is even less pronounced than of the temperature, being at most a factor of 2 over a salinity range of 0 to 36‰ (Whitehouse, 1984; McElroy *et al.*, 1989).

Table 1.2 – Range of PAH concentrations in the dissolved phase of seawater in different areas of the world (ng l^{-1}).

Local	England and Wales[1]	Tamar Estuary[2]	Baltic Sea[3]	Baltic Sea[4]	Eastern Mediterranean[5]	Tyrrhenyan Sea[6]	Alexandria Coast[7]	Western Xiamen Sea[8]	Chesapeake Bay Estuaries[9]	Nova Scotia[10]	NY-NJ Harbour Estuary[11]
N	6 – 6850	13.9	0.13 – 1.77			98		120 – 1400	5.13 – 18.8		
Ace						607		270 – 4180	0.102 – 0.21		
Ac	1 – 1740		0.09 – 0.45			32		110 – 5700	0.206 – 0.977	14 – 22	
F	1 – 1400		0.30 – 1.26		0.24	26	1.0 – 175	130 – 2290	0.607 – 0.979	10 – 18	0.76 – 2.6
P	3 – 1170	8.8	0.38 – 1.31	0.11 – 2.7	0.47	22	32 – 143	200 – 1370	0.963 – 1.09	10 – 25	0.92 – 5.6
A	1 – 157	4.9	0.03 – 0.08		0.06	17	16 – 76	170 – 1020	0.064 – 0.069	14	0.20 – 1.6
Fluo	1 – 940	10.4	1.49 – 3.93	0.33 – 6.97	0.21	19	11 – 62	90 – 2870	0.337 – 0.632	14 – 19	0.45 – 14
Py	1 – 1090	18	0.41 – 2.00	0.26 – 2.87	0.17	43	11 – 52	220 – 2660	0.103 – 0.264	12 – 41	0.40 – 16
BaA	1 – 609	15.2	0.08 – 0.25		0.06	2		280 – 1800	0.0016 – 0.016	13	0.02 – 1.6
Ch	1 – 726	3.5	0.14 – 0.37		0.14	2	13 – 83	0 – 1070	0.008		0.10 – 2.4
BbF	1 – 621	9.3	0.09 – 0.22			41		130 – 1820	0.0016 – 0.024		
BkF	1 – 250	4.2	0.04 – 0.09			19		210 – 2190	–		
BaP	1 – 909	9.1	0.02 – 0.12	0.03 – 1.50		5	16 – 82	560 – 3320	0.011		0.01 – 0.01
DBA	1 – 126		0.01 – 0.02			4		370 – 6030	0.033		0.01 – 0.01
Bper	1 – 627	83.4	0.04 – 0.12			2		410 – 1930	–		0.00 – 0.00
IP			0.05 – 0.13					960 – 5420	–		0.02 – 0.02
tPAH	1 – 10724		3.32 – 11.04	0.74 – 13.58	1.35	937	128 – 642	9090 – 26900	8.90 – 21.75	10 – 134	

[1]Law *et al.*, 1997; [2]Readman *et al.*, 1982; [3]Witt, 2002; [4]Witt & Siegel, 2000; [5]Tsapakis *et al.*, 2003; [6]Cincinelli *et al.*, 2001; [7]Nemr & Abd-Allah, 2003; [8]Maskaoui *et al.*, 2002; [9]Gustafson & Dickhut, 1997; [10]Goodarzi & Mukhopadhyay (Muki), 2000; [11]Gigliotti *et al.*, 2002.

1.1.4. Polycyclic Aromatic Hydrocarbons in sediments

The PAH concentrations in the sediments are generally much higher than in the surrounding water body (Herbes, 1976; McGroddy *et al.*, 1996; Lun *et al.*, 1998; Kowalewska, 1999; Kirso *et al.*, 2001). The importance of sediments as PAH reservoirs is well documented in natural as well as experimental systems (Baumard *et al.*, 1998a; Narbonne *et al.*, 1999). Sediment PAH concentrations in several locations in Europe are presented in Table 1.3.

Table 1.3 – PAHs concentrations in the sediments from different locations in Europe.

Location	Concentration range (ng g^{-1} dw)	Reference
Gironde Estuary – Mediterranean Sea	1.21 – 8420	Baumard *et al.* , 1998b
Mediterranean North West Coast	86.5 - 48090	Benlahcen *et al.* , 1997
Mediterranean Sea (French Coast)	1.18 – 20400	Baumard *et al.* , 1999b
Eastern Mediterranean Sea	2.2 – 1056.2	Tsapakis *et al.* , 2003
Venice Canals (Italy)	54000 – 160000	Wetzel & Van Vleet, 2003
Adriatic Sea	100 – 112000	Fabbri *et al.* , 2003
Seine River (France)	1400 – 127000	Carpentier *et al.* , 2002
Arcachon Bay – France	31.7 – 4120	Baumard *et al.* , 1999b
Oder Estuary – Baltic Coast	1693 – 7969	Kowalenska *et al.* , 1997
Southern Baltic	10 – 7000	Kowalenska & Konat, 1997
Baltic Sea (Germany and Poland Coasts)	3.96 – 22100	Baumard *et al.* , 1999b
Odra River (Poland)	< dl – 153600	Wolska *et al.* , 2003
Northern Ireland Loughs	83 – 2300	Guinan *et al.* , 2001
Caspean Sea	1 – 1600	Tolosa *et al.* , 2003

Small-scale physical processes such as local turbulence, and large-scale physical forces such as currents, which redistribute particulate material, will also affect PAHs distribution, as well as sediment mixing by burrowing organisms (bioturbation), especially near the sediment-water interface (Klamer *et al.*, 1990; Cousins *et al.*, 1999; Grossi *et al.*, 2002; Nickell *et al.*, 2003; Wheatcroft & Drake, 2003).

1.1.5. Polycyclic Aromatic Hydrocarbons in marine organisms

PAHs accumulation may occur in most marine organisms in a wide range of concentrations (Meador *et al.*, 1995). Table 1.4 presents PAH concentrations in several marine bivalve molluscs. It may be seen there that bivalves are able to accumulate high amounts of PAHs. In general, mussels (*Mytilus sp.*) and oysters (*Crassostrea gigas* and *Ostrea edulis*) present the highest PAH concentrations. Still, high concentrations were also reported for some clams, namely *Tapes semidecussata* from the Galicia Coast (1460 ng/g d.w.) and *Mercenaria mercenaria* from Rhode Island (4700 – 67800 ng/g w.w.). The wide range of concentrations seen in Table 1.4 may be the result of several variables, including different environmental PAH concentrations, time of exposure, their feeding behaviour and ability to metabolise these compounds (Meador *et al.*, 1995; Okay *et al.*, 2000).

In invertebrates, highest concentrations are generally found in lipid rich organs as the hepatopancreas (Neff, 2002). Tissue concentrations, however, appear to follow seasonal cycles, which may be related to variations in lipid content or spawning cycles (Meador *et al.*, 1995; Baumard *et al.*, 1999a).

Uptake of hydrophobic contaminants by marine organisms may proceed via several sources: water filtration through the gills, filtration of overlying or pore-water, direct contact with sediment, or ingestion of sediment and/or food particles (Spacie *et al.*, 1995). PAHs route of uptake is generally governed by their K_{ow} (Farrington, 1989; Neff & Burns, 1996). For the more soluble PAHs, the major route of uptake is generally ventilation of water through the gills (Meador *et al.*, 1995; Neff, 2002), which may, however, be affected by the water-phase composition: as stated before (section 1.1.3), a large fraction of the total "dissolved" PAHs in the

Table 1.4 – tPAH concentrations in bivalve molluscs from several places worldwide.

Species	Area	tPAH range	Reference
Tapes semidecussatus	Galicia Coast	59 – 1460 [b]	Porte *et al.* , 2000
Ruditapes phillipinarum	Venice Lagoon	2.1 – 16.3 [a]	Binelli & Provini, 2003
	Venice Lagoon	7.44 – 50.9 [b]	Nasci *et al.* , 2000
	Tyrrenian Sea	8.5 – 16.1 [a]	Binelli & Provini, 2003
	French Coast – Atlantic Ocean	22.5 [a]	Binelli & Provini, 2003
Venus gallina	The Netherlands	13.8 [a]	Binelli & Provini, 2003
	Adriatic Sea	10.9 – 24.5 [a]	Binelli & Provini, 2003
Mercenaria mercenaria	Tampa Bay	25.5 [a]	Nasci *et al.* , 1999
	Rhode Island	4700 – 67800 [a]	Pruell *et al.* , 1984
Mytilus galloprovincialis	Urdaibai estuary (Bay of Biscay)	21.0 – 64.3 [a]	Orbea *et al.* , 2002
	Galician Coast	20.8 – 202.8 [a]	Porte *et al.* , 2001a
	Barcelona harbour	336 [b]	Baumard *et al.* , 1998b
	Vendres harbour	337 [b]	Baumard *et al.* , 1998b
	Mediterranean Spanish Coast	25.1 – 82.2 [b]	Baumard *et al.* , 1998b
	Mediterranean French Coast	39.0 – 79.6 [b]	Baumard *et al.* , 1998b
	Western Mediterranean Sea	25.6 – 390 [b]	Baumard *et al.* , 1998a
Mytilus californianus	San Francisco Estuary	21 – 1093 [b]	Oros & Ross, 2005
Mytilus edulis	Northern Ireland	95 – 184 [b]	Guinan *et al.* , 2001
	Galicia Coast	42 – 2440 [b]	Porte *et al.* , 2000
	Baltic Sea	87.7 – 3880 [b]	Baumard *et al.* , 1999a
Crassostrea sp.	Urdaibai estuary (Bay of Biscay)	31.7 – 208.4 [a]	Orbea *et al.* , 2002
Crassostrea gigas	San Francisco Estuary	184 – 6899 [b]	Oros & Ross, 2005
Ostrea edulis	Galicia Coast	251 – 594 [b]	Porte *et al.* , 2000

[a] ng g^{-1} w.w.; [b] ng g^{-1} d.w.

water column are actually sorbed or complexed to DOM or colloids, reducing their bioavailability to some species. In fact, several experiments of non-ionic hydrophobic compounds, as the PAHs, accumulation by organisms have demonstrated decreases in bioconcentration in the presence of DOM and humic-like material (Farrington, 1989; Haitzer et al., 1999; Akkanen & Kukkonen, 2001; Akkanen & Kukkonen, 2003; Gourlay et al., 2003). Conversely, accumulation of the more hydrophobic PAHs is thought to proceed mainly through the ingestion of contaminated sediment or food particles (Meador *et al.*, 1995). Bioaccumulation from sediments or food usually involves an intermediate step in which PAHs desorb or are released into solution from the solid matrix (Neff, 2002). Thus, the environmental compartment in which PAHs exist will be determinant on its bioavailability (Spacie *et al.*, 1995).

PAHs sorbed to dissolved and particulate organic matter in water and sediments may still desorb from these substrates, maintaining water PAH concentrations at an equilibrium (Meyers & Qujnn, 1973; Readman *et al.*, 1982). However, during long-term contact between PAHs and sediment particles, PAHs become tightly bound to the organic phase in the sediment, reducing their bioavailability. Bioaccumulation from sediments may also be affected by sediment characteristics such as particle size and organic matter content (OM) (Borglin et al., 1996; Kukkonen & Landrum, 1996; Baumard *et al.*, 1999a; Bi et al., 2002). Usually, organisms preferentially ingest the smaller sediment particles (more enriched in OM), which may result in an increased contaminant uptake (Van der Oost *et al.*, 2003).

Different methods have been used to study contaminants bioaccumulation. The bioconcentration factor (BCF = ratio of the organism concentration of a compound versus its water concentration) is a useful first order approach for estimating biological uptake and accumulation of organic chemicals. The bioaccumulation factor (BAF = ratio of the organism concentration versus the sediment concentration) is calculated to estimate the contaminant accumulation by an organism from the sediment; and the biota sediment accumulation factor (BSAF = ratio of the lipid-normalized concentration in the tissue to the carbon-normalized sediment concentration) is based on the partitioning of the contaminant between the sediment organic carbon and the organism lipids (Spacie *et al.*, 1995). Table 1.5 presents bioconcentration and bioaccumulation factors of PAHs for several organisms. BCF values are much higher than BAF or BSAF, ranging from 2.3 for naphthalene in *Rangea cuneata* to 226000 for indene[123-*cd*]pyrene in *Mytilus edulis*. For the low molecular weight hydrocarbons (with lower log K_{ow} values), BCFs were similar for all species. However, for the higher molecular weight PAHs (higher than chrysene), *Mytilus* and *Crassostrea* presented higher BCFs than clams. Contrary to BCF, BAF and BSAF values were generally higher for the low molecular weight PAHs.

Table 1.5 – BCF, BAF and BSAF for PAHs in several organisms.

	Species	N	F	P	A	Fluo	Py	BaA	Ch	BbF	BkF	BaP	DBA	Bper	IP	tPAH	Reference
BCF	*Mercenaria mercenaria*			1974		6934	8172	16516	7335	10331		4143				4072	Bender *et al.* , 1988
	Rangea cuneata	2.3															Neff *et al.* , 1976
	Mytilus edulis	65.1	430	1020		4340	3970	20100	18000			26800	130000		226000		Neff, 2002
	Crassostrea virginica			1604		6965	8857	28846	26019	84217		19673				15190	Bender *et al.* , 1988
BAF	*Mytilus edulis*											65383					Okay *et al.* , 2000
	Mytilus edulis															0.02-52.2	Baumard *et al.* , 1999a
	Mytilus sp.			9.0-22.7	4.4-17.8	5.5-6.1	5.5-9.5	3.7-14.0	10.1-17.5	4.2-9.6		1.7-4.0	3.6-7.0	4.4-7.0	2.5-5.5		Baumard *et al.* , 1999b
BSAF	*Coullana sp.*					0.22-0.67											Lotufo, 1998
	Schizopera knabeni					0.51-0.80											Lotufo, 1998
	Arenicola marina			0.95	0.72	0.86	1.61	0.61			0.24	0.12					Neff, 2002
	Lumbriculus variegatus		5.74		5.48						5.17	2.68		1.24	4.46		Hyötyläinen & Oikari, 2004
	Mercenaria mercenaria															0.0172	Nasci *et al.* , 1999
	Mytilus edulis															0.01-0.59	Baumard *et al.* , 1999a
	Mytilus edulis					0.012-2.64	0.005-1.15	0.006-1.48	0.008-1.76	0.003-0.590							Hellou *et al.* , 2002
	Ictalurus nebulosus			2-10		2-6	2-5		1-2							1-6	van der Oost *et al.* , 2003

Several studies have demonstrated the existence of a relationship between log BCF and log K_{ow}, based on different groups of chemicals, species and exposure conditions (McElroy *et al.*, 1989; Neff, 2002). In fact, BCF/K_{ow} relationships may be affected by both biotic and abiotic factors. Biotic factors include those that are dependent on the organism, generally varying with species. These include lipid content and distribution within the animal tissues (which often varies with age, sex and phase of the reproductive cycle), feeding type, metabolic rate of the animal (which varies also with age and nutritional status of the animal), and behavioural effects of experimental exposures (Jimenez *et al.*, 1987; Meador *et al.*, 1995). Abiotic factors include temperature, salinity, and the physical form of the chemical in the water.

1.2. BIOTRANSFORMATION

Organisms have two ways of eliminating chemicals: excretion in its original form (the parent compound) or biotransformation (Van der Oost *et al.*, 2003). Release of bioaccumulated PAHs by molluscs may occur by passive elimination through equilibrium partitioning between body lipids and the external water (Neff, 2002). However, though passive elimination may be a fast process for the low molecular weight and thus more soluble PAHs, it is very slow for the less soluble compounds (Meador *et al.*, 1995). If active metabolism and excretion do not occur, these compounds may accumulate in the tissues of marine organisms (Neff, 2002). Most marine organisms are, however, able to metabolize some PAHs converting them to polar, more soluble by-products that are readily excreted (Anderson, 1985; Snyder, 2000).

Overall, PAHs metabolism involves three major steps or phases acting in a tightly integrated manner (Sheehan *et al.*, 2001). Phases I and II involve the conversion of the lipophilic, non-polar xenobiotic into a more water-soluble metabolite, which can then be eliminated more easily from the cell in Phase III (Figure 1.1).

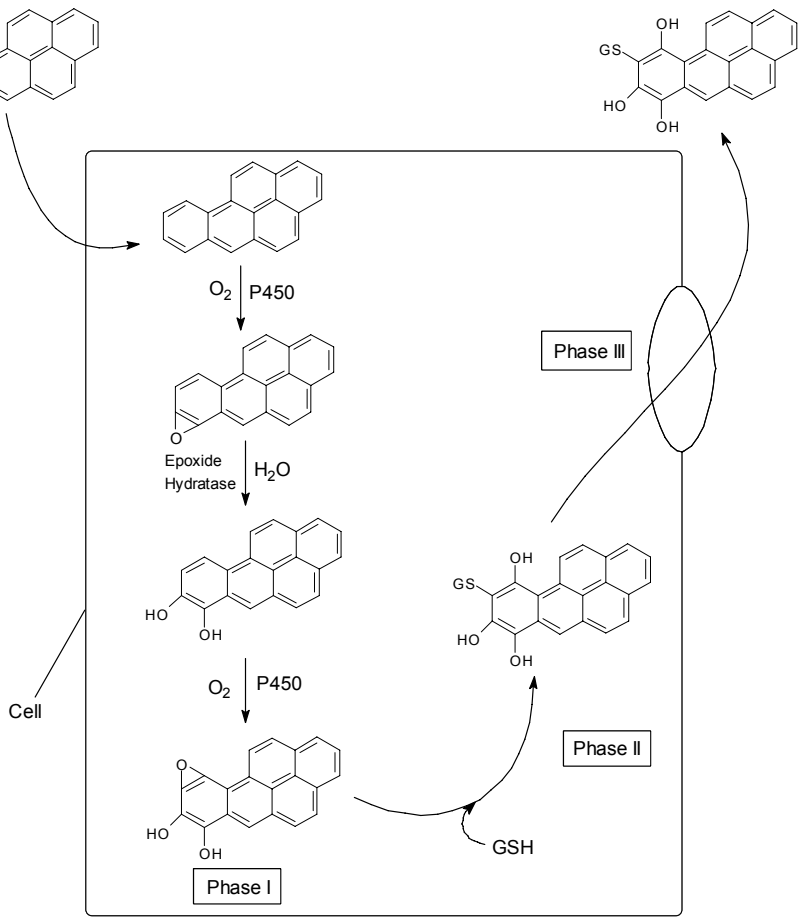


Figure 1.1 – Overview of benzo[*a*]pyrene detoxification. Adapted from Sheehan *et al.* (2001).

Phase I involves the oxidation of the organic chemical with molecular oxygen, catalysed by monooxygenases (Lee, 1981; Livingstone *et al.*, 1985; Goksøyr & Förlin, 1992; Solé *et al.*, 1994). These transformations rely on the action of enzymatic systems such as the cytochrome P450-dependent monooxygenase or mixed-function oxygenase (MFO) system, flavoprotein monooxygenase system, and epoxide hydratase and flavoprotein reductases.

The enzymes responsible for phase I metabolism are generally associated with the membrane of the endoplasmic reticulum of the liver (vertebrates) or hepatopancreas (invertebrates) cells (Michel *et al.*, 1993). Phase I biotransformation products are however more hydrophilic than the parent ones and move to the cytosol, where the enzymes responsible for phase II metabolism are located.

The MFO system is present in many marine invertebrate species, as arthropods (crustaceans), annelids (polychaetes), cnidarians, molluscs, porifera, platyhelminths, and echinoderms (Snyder, 2000). However, it appears to be much less inducible by xenobiotics in these organisms than in marine vertebrates including fish (Solé *et al.*, 1994).

Phase I mechanism is illustrated in more detail in Figure 1.2, for benzo[*a*]pyrene, where the microsomal electron transport reactions involved in the metabolism are shown (Lee, 1981).

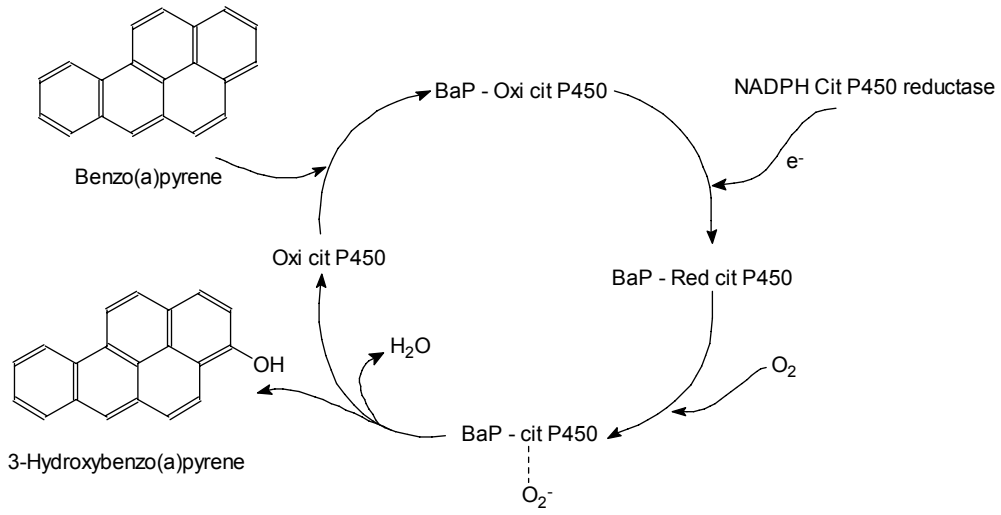


Figure 1.2 – Benzo[*a*]pyrene hydroxylation by the cit P450 oxygenase system. Benzo[*a*]pyrene binds first to oxidized cytochrome P450 (Fe^{3+}) and iron is reduced in the resulting complex to cytochrome P450 (Fe^{2+}) which than interacts with oxygen producing 3-hydroxybenzo[*a*]pyrene, and regenerating the oxidized cytochrome P450. The reduction of the substrate-oxidized cytochrome P450 complex involves the donation of two electrons from NADPH and is carried by NADPH cytochrome P450 reductase. The superoxide anion (O_2^-) is formed during this reaction participating in the hydroxylation of the substrate. Adapted from Lee (1981).

Benzo[*a*]pyrene hydroxylase (BPH) is one of the Cyt P450 enzyme families involved in the PAHs metabolism phase I (Buhler & Williams, 1989; Shaw *et al.*, 2004). BPH, which is also called aryl hydrocarbon hydroxylase (AHH), catalyzes the formation of arene oxides that can be auto-oxidized to form phenols or quinones (Michel *et al.*, 1993). This oxidation results in the production of metabolites with mutagenic and carcinogenic properties (Buhler & Williams, 1989). Several studies (Table 1.6) have demonstrated the occurrence of BPH in bivalves as the mussel *M. galloprovincialis* (Michel *et al.*, 1993; Michel *et al.*, 1994; Porte *et al.*, 2001) and *M.*

edulis (Solé *et al.*, 1998) and in clams *M. mercenaria* (Nasci *et al.*, 1999) and *T. phillipinarum* (Nasci *et al.*, 2000).

Table 1.6 – Benzo[*a*]pyrene hydroxylase activity (fluorescence units mg^{-1} prot) range in different bivalve species.

Species	Area	Tissues	BPH (fu mg^{-1}prot)	Reference
Tapes philippinarum	Venice Lagoon	DG	0.7 - 1.5	Nasci *et al.* , 2000
Mercenaria mercenaria	Tampa Bay, Florida	DG	0.069 - 0.242	Nasci *et al.* , 1999
Mytilus galloprovincialis	Mediterranean Sea	DG	2.5 - 24.5	Michel *et al.* , 1994
	Galician Coast	DG	44.7 - 63.2	Porte *et al.* , 2001

Phase II metabolism is conducted by conjugative enzymes, such as glutathione S-transferase (GST), UDP-glucoronyl transferase, UDP-glucosyl transferase, sulphotransferase and amino acid conjugases, which will attach a large polar moiety (glutathione, sulphate, glucoronide, amino acid, etc.) to the functional group introduced into the xenobiotic in Phase I metabolism.

GST is a multigene superfamily of dimeric, multifunctional, primarily soluble enzymes, found mainly in the cytosol (Sheehan *et al.*, 2001; Van der Oost *et al.*, 2003). It catalyzes the conjugation of electrophilic compounds (or phase I metabolites) with GSH (Sheehan *et al.*, 2001). Although it may also have peroxidase and isomerase activities, GST can inhibit the Jun N-terminal kinase (thus protecting cells against H_2O_2-induced cell death), and they are able to bind non-catalytically to a wide range of endogenous and exogenous ligands (Sheehan *et al.*, 2001). GST activity is present in different bivalves (Table 1.7) such as mussels *M. edulis* (Lyons *et al.*, 2003) and *M. galloprovincialis* (Akcha *et al.*, 2000) and clams *Ruditapes decussatus* (Hoarau *et al.*, 2001; Hoarau *et al.*, 2004) and *R. phillipinarum* (De Luca-Abbott *et al.*, 2005).

Table 1.7 – Glutathione S-transferase activity range in different bivalve species.

Species	Area	Tissues	GST (μmol min^{-1} mg^{-1} prot)	Reference
Ruditapes decussatus	Thau Lagoon (Mediterranean)	Whole	0.75	Hoarau *et al.* , 2001
		Gills	3.3 - 5.2	Hoarau *et al.* , 2004
		DG	1.0 - 1.4	
Ruditapes philippinarum	Venice Lagoon	DG	10.5 - 39.5	Livingstone *et al.* , 1995
	Hong Kong	Gills	0.35 - 0.90	De Luca-Abbott *et al.* 2005
		DG	0.40 - 1.3	
Mytilus galloprovincialis	Thau Lagoon (Mediterranean)	Gills	0.15 - 0.17	Akcha *et al.* , 2000
Perna viridis	Hong Kong	Gills	0.0958 - 0.182	Cheung *et al.* , 2001
		DG	0.0579 - 0.114	
		Gills	0.04 - 0.17	De Luca-Abbott *et al.* 2005
		DG	0.04 - 0.29	

Phase III metabolism is conducted by enzymes (peptidases, hydrolases and β-lyase), which catalyse the catabolism of the conjugated metabolites to form easily extractable products.

The metabolites originated in phases I and II of the PAHs metabolism are generally considered detoxification products (Varanasi *et al.*, 1989). However, some of these, namely the bay-region-diol-epoxides, are capable of inducing cytotoxicity and mutation in cells (Varanasi *et al.*, 1989) leading to the formation of DNA adducts (Canova *et al.*, 1998). DNA adducts are mutagenic and carcinogenic products which may induce tumours in the tissues (Bartsch, 1996). Besides formation of these reactive metabolites, PAHs metabolism by the MFO system may also produce metabolites capable of undergoing redox cycling, leading to oxyradical generation (Lemaire *et al.*, 1994). For example, in the fish brown bullhead, BaP quinones represent a major portion of phase I oxidation products (Sikka *et al.*, 1990; Yuan *et al.*, 1997). Quinones may undergo either redox cycling or phase II conjugation (Sjölin & Livingstone, 1997).

Redox cycling (Figure 1.3) is a secondary metabolic pathway in which the organic xenobiotic is reduced by flavoproteins, transforming it into a radical metabolite capable of directly reacting with molecular oxygen, producing superoxide anion and regenerating the parent compound (Dicker & Cederbaum, 1991).

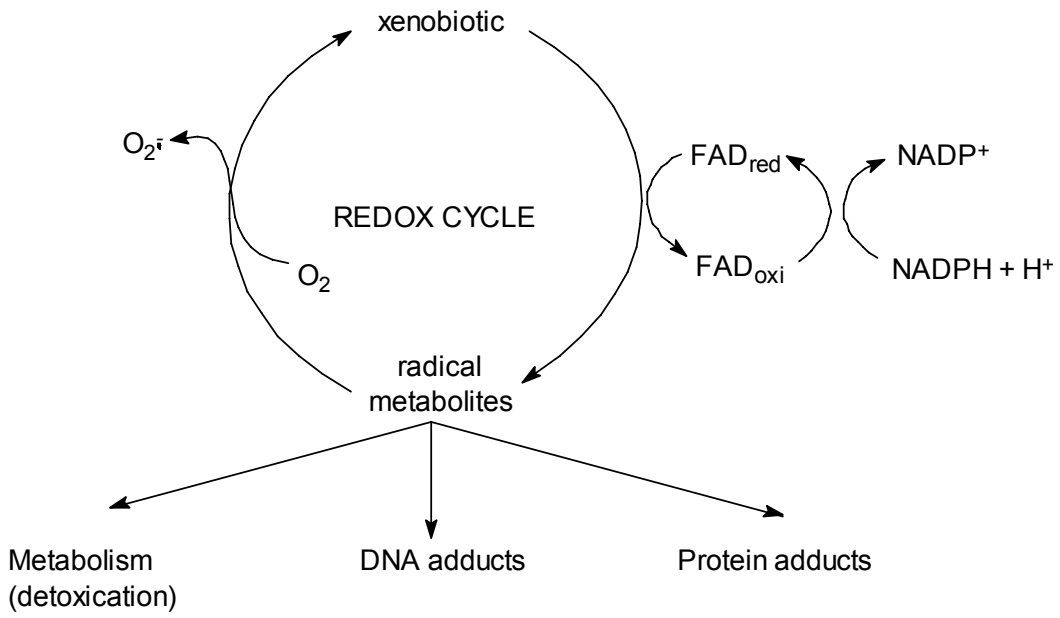


Figure 1.3 – Redox cycling of xenobiotic compounds. Adapted from Di Giulio *et al.* (1995).

This cycle may repeat itself producing superoxide anions until the radical metabolite is either adducted to an endogenous macromolecule (e.g., DNA or an enzyme) or conjugated and eliminated (detoxified), hence the importance of redox cycling as a bioactive process (Di Giulio *et al.*, 1995). Damage begins to accumulate when formation of active oxygen species exceeds the capacity of the organism to neutralize them (Halliwell & Aruoma, 1991; Sjölin & Livingstone, 1997). This includes mutations that may eventually leads to cancer formation.

1.3. OXIDATIVE STRESS

As mentioned previously (1.2.) the metabolism of xenobiotics, by microsomal enzyme systems, can form free radicals, which can mediate tissue injury. In this section other cellular sources of free radicals and other non-radical reactive species as well as their reactions with cellular constituents will be summarily described. The products resulting from lipid oxidation and the defence systems against oxidative damage will be described in more detail, since they were used in this work as biomarkers of oxidative stress.

1.3.1. Reactive Oxygen and Nitrogen Species

Free radicals, molecules containing typically one or more unpaired electrons, are continually being formed in small amounts by normal metabolism processes. Due to the ubiquity of dioxygen and its ability to accept electrons, oxygen free radicals, such as the superoxide anion radical (O_2^{-}•), hydroperoxyl radical (HO_2•), hydroxyl radical (•OH) and peroxyl (ROO•) and alcoxyl (RO•) radicals are particulary important. In addition, non-radical derivatives of O_2, such as hydogen peroxide (H_2O_2) and synglet oxygen ($^1O_2$), are also formed and the set of all these species are collectively termed reactive oxygen species (ROS).

More recently it was discovered that an important nitrogen radical is also formed in organisms, the nitric oxide radical (•NO), which has important physiological functions (Ignarro, 1991). Nitric oxide can react with O_2 to form the more reactive radical nitrogen dioxide (•NO_2) and with O_2^{-}• to give peroxynitrite ($ONOO^{-}$), a non-radical but a very oxidizing species (Radi *et al.*, 2001). These nitrogen derivatives are designated reactive nitrogen species (RNS). Carbon- and sulfur-centerd radicals may also occur in biological systems and are important in initiating and/or propagating chain reactions.

Free radicals are generally very reactive and participate in hydrogen abstraction, electron transfer and addition and dispropotionation reactions. But, contrary to what was initially assumed, some free radicals are highly beneficial to the organisms and it has recently become clear that they play an important role as modulators of intracellular signal transduction (Lander, 1997; Dröge, 2001; Cadenas, 2004). When they are produced in excess, however, they can damage all major classes of biomolecules, lipids, proteins, DNA and carbohydrates. Antioxidant systems exist in organisms to protect against reactive species but, when a disturbance in the prooxidant-antioxidant balance occurs in favour of the former and leading to potential damage, a situation of oxidative stress results (Sies, 1985).

There are a number of intracellular sources for free radicals (Freeman & Crapo, 1982; Kehrer, 1993). They are produced through the catalytic activity of various soluble and membrane-bound enzymes (e.g. xanthine oxidase, aldehyde oxidase, tryptophan dioxygenase and NADPH oxidases). The mitochondrial electron transport system is an important source of $O_2^{-}\bullet$ and can involve NADH-ubiquinone and ubiquinol – cytochome c oxireductases. Many soluble components of the cytosol, capable of undergoing oxidation-reduction reactions are important contributors to intracellular free radical (e.g. thiols, hydroquinones, reduced flavins, haem proteins). In cells free radicals can also be derived following exposure to ionizing radiation and during exposure to drugs and xenobiotics, which can be metabolized to radicals by microsomal enzyme systems. Peroxisomes possess high concentrations of oxidases (e.g. D-amino acid oxidase, urate oxidase, and fatty acyl-CoA oxidase), which have been shown to reduce O_2 directly to H_2O_2.

$O_2^{-}\bullet$ generation may lead to the production of other reactive oxygen species including H_2O_2 via dismutation (1)

$$2\,O_2^{-} + 2\,H^{+} \rightarrow H_2O_2 + O_2 \qquad (1)$$

and •OH via the Haber-Weiss reaction (2) (Sjölin & Livingstone, 1997)

$$O_2^{-} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^{-} + O_2 \qquad (2)$$

This reaction is thermodynamically favourable but kinetically very slow (Halliwell & Aruoma, 1991) and takes place at significant rates only in the presence of suitable chelated transition metals such as iron or copper (3), via the following half-reactions, the sum of which is the Haber-Weiss reaction (2):

$$O_2^{-\bullet} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \qquad (3)$$

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^{-} + Fe^{3+} \qquad (4)$$

The second half-reaction (4) is the Fenton reaction (Halliwell & Aruoma, 1991). In addition, through a Fenton type reaction, preformed lipid hydroperoxides (ROOH) are decomposed to form the alkoxyl radicals ($RO^{\bullet}$), strong oxidants, which can propagate the chain reaction of lipid peroxidation (Svingen *et al.*, 1979; Cadenas, 1989; Halliwell & Gutteridge, 1999) or react with other cell constituents. In order to be an effective catalyst in those radical reactions, iron has to be present in an ionic or "free" form. Usually iron is safely sequestered in proteins that normally bind iron hindering or preventing its action in catalysing radical reactions. Iron, however, can be released from those proteins at low pH (Baker & Gebick, 1986) as a result of protein damage produced by peroxides (Gutteridge, 1986) or by reductive mobilization by $O_2^{\bullet-}$ (Thomas *et al.*, 1985). The highly potent •OH will react indiscriminately with most biological molecules, including proteins, lipids and DNA. Thus, proliferation of $O_2^{-}\bullet$, H_2O_2, •OH and oxidative products (e.g. lipid peroxides) can result in a spreading web of oxidative damages, whose cumulative effects may be the proximate cause of cell death (Borg *et al.*, 1978; Sies, 1986; Winston & Di Giulio, 1991).

1.3.2. Oxidative Damage

As mentioned previously, proteins, lipids and DNA are all targets for oxidative damage, and a vast array of changes has been identified that could provide a mechanistic explanation for this damage. The changes believed to be most likely responsible for injury include: damage to Ca^{2+} and other ion transport systems responsible for maintenance of normal ion gradients, activation/deactivation of various enzyme systems, activation of poly(ADP) polymerase, changes in gene expression, and depletion of ATP and NAD(P)H (Kehrer, 1993; Van der Oost *et al.*, 2003).

A. Protein Oxidation

Oxidation of proteins by ROS can lead to oxidation of amino acid residue side chains, cleavage of peptide bonds, and formation of covalent protein-protein cross-linked derivatives (Stadtman & Berlett, 1997). Protein oxidation can lead to a loss of critical sulfhydryl groups and to modifications of amino acids leading to the formation of carbonyl and other oxidized moieties (Stadtman & Berlett, 1997). The ROS-mediated modification of proteins may lead to loss of biological function and to conversion of the proteins to forms that are rapidly degraded by endogenous proteases.

B. DNA Oxidation

DNA itself is a very stable molecule, but oxidative stress greatly accelerates DNA damage. Superoxide, nitric oxide or H_2O_2 do not appear to react with DNA. By contrast exposure of DNA to the highly reactive •OH generates a large amount of products. Fenton chemistry is undoubtedly the main source of •OH and, therefore, the transition-metal ions that convert H_2O_2 into •OH must be very close to DNA (site specific generation of •OH). Oxidation of DNA, by oxidants, can result in damage to all four bases and the deoxyribose. Attack to DNA bases leads to as many as 50 base alterations. One of the most abundant and easily measured products of DNA oxidation is 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG), which has received more attention because of its highly mutagenic nature and, therefore, may be involved in the initiation of cancer (Canova et al., 1998; Ploch et al., 1999).

C. Lipid Peroxidation

The most susceptible biomolecules to free radical attack are polyunsaturated fatty acids (PUFA), which are major constituents of phospholipids and cholesterol of all cell membranes (Di Giulio *et al.*, 1995). The methylene interrupted ($–CH_2–$) double-bond structure of the PUFA

makes them particularly susceptible to hydrogen abstraction, leading to a stable lipid free radical ($R^{\bullet}$) (Figure 1.4).

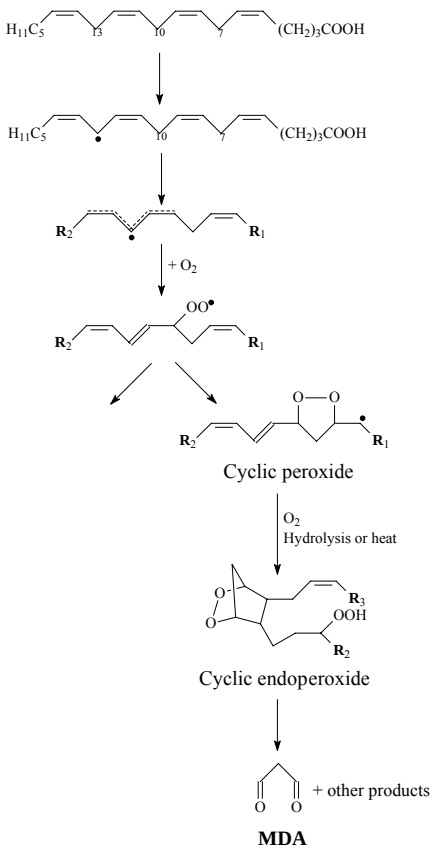


Figure 1.4 – Scheme of arachidonic acid lipid peroxidation (C20:4). Initial abstraction of an allylic H from at one of three (C-13) positions is shown. H can also be abstracted at C-10 or C-7, giving several other peroxide end-products. R_1 = CH_2-CH-CH-$(CH_2)_3COOH$, R_2 = C_5H_{11}, R_3 = $(CH_2)_3COOH$. Adapted from Halliwell & Gutteridge (1999).

Initiation is the term used to describe the abstraction of the hydrogen atom from a PUFA, which can be achieved by •OH and HO_2• radicals and chelated iron-oxygen such as ferryl ($Fe^{2+}O$) or perferryl ($Fe^{2+}O_2$), leading to the propagating chain reactions characteristic of lipid auto-oxidation. Isomerisation of the fatty acid free radical then occurs spontaneously by the arrangement of the double bonds to alternate single and double bond forming conjugated dienes. This is followed by oxygen attack to form a peroxyl radical $ROO^{\bullet}$ ($k \approx 10^8$-$10^9 M^{-1}s^{-1}$), which can then, in a slow reaction ($k \approx 10^1$-$10^2 M^{-1}s^{-1}$), abstract a further hydrogen atom from another unsaturated fatty acid with the formation of lipid hydroperoxides (Cadenas, 1989; Forni, 1990).

This is the propagation stage of lipid peroxidation. The carbon radical formed can react with O_2 to form another peroxyl radical and so the chain reaction of lipid peroxidation can continue.

A single initiation event can lead to formation of multiple molecules of peroxide as result of the chain reaction. In addition the initial H abstraction from the PUFA can occur at different points on the carbon chain. For example the initial H abstraction from arachidonic acid can occurs at C-7, C-10 and C-13 and just one of the six possible structures is shown in Figure 1.4 (Halliwell & Gutteridge, 1999). The propagation reactions can continue indefinitely or may be terminated either by reactions between two radicals or by the reaction between a radical and an antioxidant molecule AH.

$$ROO^{\bullet} \text{ (or } R^{\bullet}) + R^{\bullet} \rightarrow ROOR \text{ (or } R_2)$$

$$ROO^{\bullet} \text{ (or } R^{\bullet}) + AH \rightarrow ROOH \text{ (or RH)} + A^{\bullet}$$

Lipid peroxidation gives rise to a wide variety of oxidation products which can be monitored to assess lipid peroxidation, either *in vitro* or *in vivo* (Moore & Roberts II, 1998). Particularly important are reactive aldehydes, such as malondialdehyde and 4-hydroxyquenals, which can cause cross-linking and polymerisation of membrane components. In fact, because of the hydrophobic nature of the lipid radicals, most of the reactions will take place with membrane-associated molecules. After peroxidation of membrane fatty acids, the presence of shortened-chain fatty acids containing R-OOH, R-COOH, R-CHO and R-OH groups may seriously affect membrane permeability and microviscosity (Freeman & Crapo, 1982). This can alter intrinsic membrane properties such as deformability, ion transport, enzyme activity and the aggregation state of cell surface determinants (Freeman & Crapo, 1982).

Certain compounds such as PCBs, PAHs, some nitroaromatic compounds and various metals can increase lipid peroxidation (Livingstone *et al.*, 2000; Shaw *et al.*, 2004). See Table 1.8 for LPO levels in different bivalves.

1.3.3. Antioxidant Defences

ROS produced in biological systems are detoxified and controlled by antioxidant defences, which are generally present in all kinds of animal species including aquatic organisms, namely, in polychaetes (Blum & Fridovich, 1984), echinoderms (Gamble *et al.*, 1995), bivalves (Solé *et al.*, 1994; Cossu *et al.*, 2000; Orbea *et al.*, 2002; Camus *et al.*, 2003) and fish (Ploch *et al.*, 1999; Livingstone, 2001; Stephensen *et al.*, 2002; Li *et al.*, 2003; Van der Oost *et al.*, 2003).

Antioxidant systems are mainly composed of water and fat-soluble low molecular weight free radical scavengers and specific antioxidant enzymes (Livingstone, 2001). The non-enzymatic low molecular weight antioxidants include: the water-soluble reduced glutathione (GSH) (Di Giulio *et al.*, 1995; Van der Oost *et al.*, 2003), ascorbic acid (vitamin C) (Di Giulio, 1991), and the lipid soluble-carotenenes (including β-carotene), which is the precursor (forerunner) of vitamin A and α-tocopherol (vitamin E), which offer protection against some ROS and lipid peroxidation (Sies, 1986; Thomas & Reed, 1990). The major antioxidant enzymes are superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidases (GPx) (Figure 1.5). These enzymes structure and function will be described further on, since they were the subject of the present study.

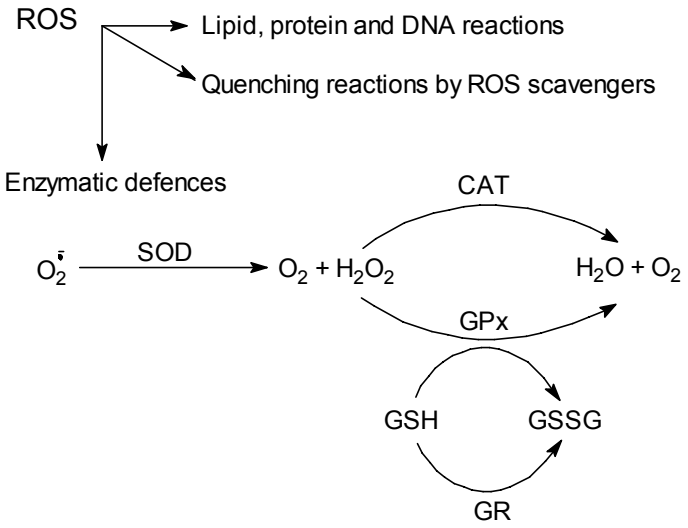


Figure 1.5 – Scheme of free radical defence mechanisms. Adapted from Di Giulio *et al.* (1995).

Other antioxidant enzymes include ascorbate peroxidase (AsPx), glutathione reductase (GR) and DT-diaphorase (DTD). AsPx exists mainly in chloroplasts, where it detoxifies cells reducing H_2O_2, using ascorbate as a co-factor (Di Giulio *et al.*, 1995). GR, although not directly involved in antioxidant defence as the other enzymes, has a chief role in maintaining the GSH/GSSG homeostasis under oxidative stress conditions (Van der Oost *et al.*, 2003). DTD catalyzes the two-electron reduction of quinones to the corresponding hydroquinone, thus providing antioxidant protection by diverting quinone metabolism away from one-electron reduction to semiquinone radicals (Di Giulio *et al.*, 1995).

A. Superoxide Dismutases

SODs (EC 1.15.1.1) are a group of metalloenzymes that catalyze the dispropotionation of superoxide to give H_2O_2 and O_2 (5), in a second order reaction with a rate constant of $2x10^9$ M^{-1} s^{-1}.

$$2O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (5)$$

SODs are considered to play a fundamental antioxidant role, whose importance is indicated by their presence in all aerobic organisms (Van der Oost *et al.*, 2003). Specific isoenzymes are typically found in cytosol, mitochondria and chloroplasts (Di Giulio *et al.*, 1995). Four classes of SODs have been identified, containing either a dinuclear Cu/Zn or mononuclear Fe, Mn or Ni as cofactors (Matés, 2000). Bacteria contain both manganese (Mn-SOD) and iron (Fe-SOD) forms of SOD. Fe-SOD also occurs in some plant species. Cu/Zn-SOD is found in plants, animals and fungi (Matés, 2000).

Cu/Zn-SODs are generally found in the cytosol (Cannio *et al.*, 2000). They constitute a class of enzymes conserved throughout evolution, which usually have two identical subunits of about 32 kDa, each containing a metal cluster, the active site, constituted by a copper and a zinc atom bridged by a common ligand: His 61 (Matés, 2000). Inactivation of the Cu/Zn-SOD by H_2O_2 is the consequence of several sequential reactions. First, reduction of the active site Cu(II)

to Cu(I) by H_2O_2; then oxidation of the Cu(I) by a second H_2O_2, thus generating a powerful oxidant, which may be Cu(I)O, Cu(I)OH or Cu(III); and finally oxidation of the histidine, causing loss of SOD activity (Liochev *et al.*, 1998). Immunolocalization studies have placed Cu/Zn SOD preferentially in the apex of epithelial cells of *Mytilus galloprovincialis* digestive cells although it could also be found in other cell compartments such as nucleus and peroxisomes (Orbea *et al.*, 2000).

Mn-SOD is a homotetramer (96 kDa) containing one manganese atom per subunit. The oxidation state of the Mn atom varies between Mn(III) and Mn(II) during the two step dismutation of superoxide. In eukaryotic cells, Mn-SOD is strictly a mitochondrial enzyme located in the inner membrane and synthesized by nuclear genes. It is controlled by oxygen or one of its metabolites and a lowered activity is generally a consequence of lower amounts of enzyme protein and its mRNA (Matés, 2000). In *Mytilus galloprovincialis*, Mn-SOD was restricted to mitochondria (Orbea et al., 2000).

The activity of SOD in several invertebrates is presented in Table 1.8.

B. Catalase

Catalase (EC 1.11.1.6) is a tetrameric haemic enzyme consisting of four identical tetrahedrally arranged subunits of 60 kDa each. Therefore, it contains four ferriprotoporphyrin groups per molecule, and its molecular weight is about 240 kDa. Catalase (CAT) reacts with H_2O_2 to form water and molecular oxygen (6); and with proton donors (methanol, ethanol, formic acid, phenol, etc.) using 1 mole of peroxide in another type of peroxidase activity (7):

$$2\,H_2O_2 \rightarrow 2\,H_2O + O_2 \qquad (6)$$

$$ROOH + AH_2 \rightarrow H_2O + ROH + A \qquad (7)$$

H_2O_2 is enzymatically catabolized in aerobic organisms by CAT and several peroxidases. Even though CAT is not essential for some cell types under normal conditions, it protects cells from hydrogen peroxide generated within them, playing an important role in the acquisition of

tolerance to oxidative stress in the adaptive response of cells (Hunt *et al.*, 1998). It is one of the most efficient enzymes known: it cannot be saturated by H_2O_2 at any concentration (Lledías *et al.*, 1998).

CAT is localized in the peroxisomes of most cells and is involved in fatty acid metabolism (Van der Oost *et al.*, 2003). In mussels *Mytilus galloprovincialis*, CAT was immunolocalized in the duct and stomach epithelia (Orbea *et al.*, 2000). See Table 1.8 for CAT activities in different bivalves.

Table 1.8 – Antioxidant enzyme activities and LPO levels in different bivalve species.

Species	Area	Tissues	Cyt SOD (U mg^{-1} prot)	Mit SOD (U mg^{-1} prot)	CAT (mmol min^{-1} mg^{-1} prot)	T GPx (nmol min^{-1} mg^{-1} prot)	Se GPx	LPO (nmol g^{-1} prot)	Reference
Tapes semidecussata	Ebro Delta	DG	14.6±0.8		4.5±0.1		2.9±0.4		Solé *et al.* , 1994
Ruditapes decussatus	Ria Formosa	Gills	35 - 48	7 - 17		5.5 - 10	4 - 8.5	400 - 580	Geret *et al.* , 2003
	Ria Formosa	DG	20 - 23	3 - 4		5 - 13	3 - 4.2	720 - 1050	
Tapes philippinarum	Venice Lagoon	DG	0.51 - 0.64		9.66 - 11.21		0.985 - 413.5		Nasci *et al.* , 2000
Mercenaria mercenaria	Tampa Bay, Florida	DG	0.51 - 0.57		9.15 - 29.02				Nasci *et al.* , 1999
Mytilus galloprovincialis	Ebro Delta	DG	12.6±0.3		3.4±0.1		3.3±0.1		Solé *et al.* , 1994
	Venice Lagoon	DG	0.88 - 2.36		0.63 - 3.65				Nasci *et al.* , 1998
	Ebro Delta	DG	6.5 - 13.0		2.5 - 4		2.7 - 3.8		Solé *et al.* , 1995
	Venice Lagoon	DG	0.634 - 0.658		1.6 - 1.8				Livingstone *et al.* , 1995
	Bay of Biscay	DG	16.99 - 28.38		0.34 - 1.43		8.22 - 9.72		Orbea *et al.* , 2002
Unio tumidus	French rivers	Gills					47±1	2600±900	Cossu *et al.* , 2000
	French rivers	DG					83±1	5200±3400	
Perna viridis	Hong Kong	Gills				0.707 - 8.57		651.5 - 2420.8	Cheung *et al.* , 2001
	Hong Kong	DG	14.919 - 49.557			2.37 - 8.92		6920 - 14397	
Crassostrea sp.	Bay of Biscay	DG	22.83 - 31.65		2.10 - 2.66		9.13 - 16.95		Orbea *et al.* , 2002
Crassostrea gigas	Ebro Delta	DG	10.7±0.3		4.1±0.2		5.9±0.3		Solé *et al.* , 1994
Ostrea edulis	Ebro Delta	DG	9.6±0.3		5.7±0.3		3.5±0.1		
Saccostrea cucullata	Hooghly Estuary, India	DG	5.8 - 15.4		2.9 - 8.5	3.2 - 6.1			Niyogi *et al.* , 2001

C. Glutathione Peroxidases

Peroxidases are enzymes that reduce a variety of peroxides to their corresponding alcohols. While CAT employs one molecule of H_2O_2 as an electron donor in the reduction of another H_2O_2 molecule, peroxidases use other reductants. One of the most important peroxidases is the selenium-dependent glutathione peroxidase (EC 1.11.1.19), a tetrameric cytosolic enzyme, each protein subunit containing one atom of selenium at its active site that uses GSH as a cofactor (cosubstrate) (8). This Se dependent GPx catalyzes the metabolism of H_2O_2 to water, involving a concomitant oxidation of reduced GSH to its oxidized form (GSSG).

$$\mathrm{ROOH} + 2\,\mathrm{GSH} \rightarrow \mathrm{ROH} + \mathrm{GSSG} + \mathrm{H_2O} \qquad (8)$$

There are also other Se independent GPx, formerly called glutathione S-transferases, that catalyzes the reduction of hydroperoxides (ROOH) also using GSH. The ratios of reduced to oxidized glutathione (GSH/GSSG) in normal cells are high and the reduction of GSSG back to GSH is achieved by glutathione reductase enzyme, which catalyses the reaction:

$$\mathrm{GSSG} + \mathrm{NADPH} + \mathrm{H^+} \xrightarrow{GR} 2\,\mathrm{GSH} + \mathrm{NADP^+} \qquad (9)$$

The glutathione redox cycle is a major source of protection against low levels of oxidant stress, whereas CAT becomes more significant in protection against severe oxidant stress (Yan & Harding, 1997). In animal cells, the principal antioxidant enzyme for the detoxification of H_2O_2 has for a long time been considered to be GPx, as CAT has a much lower affinity for H_2O_2 than GPx (Izawa *et al.*, 1996). GPx is also considered to play an important role in protecting membranes from damage due to lipid peroxidation, since its major function is the termination of radical chain propagation by quick reduction to yield further radicals (Lawrence & Burk, 1976; Matés, 2000; Van der Oost *et al.*, 2003).

Immunolocalization studies indicated that GPx is in the digestive gland of *Mytilus galloprovincialis* (Orbea *et al.*, 2000). See Table 1.8 for GPx activities in different bivalves.

1.4. BIOMARKERS

It has been fully recognised in the last years that environmental risk assessment cannot be based solely on chemical analysis of environmental samples since this approach is not able to provide any indication on the biological effects of contaminants on biota (Solé, 2000a). The need to detect and assess the effects of contaminants at low concentrations and in complex mixtures has led to the development of a wide range of indicators of exposure to and effects of contaminants in field-exposed organisms (Solé, 2000a; Lionetto *et al.*, 2003).

Biomarkers were originally defined as any biochemical, histological or physiological alterations or manifestations of environmental stress (NRC, 1987; Hyne & Maher, 2003). Different types of biomarkers have been extensively studied in order to provide an efficient tool for environmental quality assessment (Livingstone *et al.*, 2000). According to Livingstone *et al.* (2000), biomarkers should: (i) provide a temporal and spatial integrated measure of bioavailable contaminants; (ii) demonstrate the causality through mechanistic understanding of the underlying process; (iii) identify the importance of different routes of exposure by application to organisms from different habitats and different trophic levels; (iv) detect exposure to readily metabolisable contaminants, such as PAHs; (v) have the potential through integration into a suite of measurements at different levels of biological organization (molecular through community) to be predictors of long-term ecological effects, i.e. they are prognostic of events if action is not taken.

Biomarkers are classified as specific or general biomarkers (Livingstone *et al.*, 2000). Specific biomarkers indicate precisely to what contaminant or class of contaminants the organism has been exposed to. General biomarkers do not give information about the exact cause of a given response, though they indicate that the organism is under a stressful situation (Walker

et al., 1996; Livingstone *et al.*, 2000). Under this classification, biomarkers may be further classified depending on their significance (e.g. biomarkers of exposure, effect, stress or susceptibility) (de Lafontaine *et al.*, 2000). Exposure biomarkers do not have a direct relationship to a mechanism of toxicity and they do not give information of the degree of adverse effect of the contaminant therefore indicating only that the organism has been exposed to contaminants (Walker *et al.*, 1996; Hyne & Maher, 2003). Effect biomarkers, are related to changes at cellular or molecular levels, which indicate that the organism was able to respond in a specific way to the exposure of the contaminant. Biomarkers of susceptibility indicate the inherent or acquired ability of an organism to respond to the exposure to a specific contaminant (Cullen & Redlich, 1995). These include genetic factors and changes in receptors, which alter the susceptibility of an organism to that exposure (Van der Oost *et al.*, 2003). Biomarkers of stress are non-specific biomarkers that indicate that the organism has been exposed to a contaminant, which is capable of inducing stress.

Several biomarkers have been measured in different marine organisms, particularly in fish and bivalve molluscs. Most bivalve molluscs, as mussels and clams, exhibit a series of biochemical responses to environmental contaminants (den Besten, 1998; Nasci *et al.*, 1999; Porte *et al.*, 2001). These biomarkers are summarized in Table 1.9 for different bivalves as a response to organic contaminants.

Due to the environmental concern arising from PAHs toxicity to marine organisms, several molecular responses have been studied to identify biomarkers of PAHs exposure. Among them, is cytochrome P450 system induction (Solé *et al.*, 1998; Nasci *et al.*, 1999; Peters *et al.*, 1999; Porte *et al.*, 2001), phase II enzymes activity (Akcha *et al.*, 2000; Hoarau *et al.*, 2001; Sheehan *et al.*, 2001; Orbea *et al.*, 2002), antioxidant enzymes activity (Solé *et al.*, 1994; Niyogi *et al.*, 2001a; De Luca-Abbott *et al.*, 2005) and lipid peroxidation (Porte *et al.*, 1996; Cossu *et al.*, 2000; Shaw *et al.*, 2004).

The enzymatic activity of benzo[*a*]pyrene hydroxylase (BPH) is generally considered a "biomarker of exposure" (Hyne & Maher, 2003). In relation to PAHs, increased BPH activity was observed in the digestive glands of mussels *Mytilus galloprovincialis* exposed in the laboratory to benzo[*a*]pyrene (50 mg/kg dry weight mussel) (Akcha *et al.*, 2000) and also of clams *Mercenaria mercenaria* transplanted to a PAHs contaminated environment in Tampa Bay (Nasci *et al.*, 1999).

GSTs may also be induced by exposure to organic xenobiotics, although with a much less marked response than for the MFO system (Livingstone, 1998). GST is generally considered as an "effect biomarker" (Solé, 2000a). GST induction was observed in whole tissues of clams *Ruditapes decussatus* exposed to benzo[*a*]pyrene (1.5 – 3 μg l^{-1}). Similarly, increased GST activity was observed in the digestive gland of clams *T. decussata* from an oil contaminated site in the Galician Coast (Porte *et al.*, 1996) and in the gills and digestive gland of mussels *Perna viridis* transplanted to PAHs contaminated sites in Hong Kong (Cheung *et al.*, 2001).

Table 1.9 – Antioxidant enzymes, BPH, GST and LPO in the gills and digestive gland of different bivalves as a response to organic contamination.

Parameter	Species	Tissue	Local	Chemical	Study	Effect	Reference
SOD	*Tapes phillipinarum*	DG	Venice Lagoon	PAHs, PCBs, DDTs	Transplantation	—	Nasci *et al.* , 2000
	Tapes decussata	DG	Galician Coast	oil contamination	Seasonal variation	↑	Porte *et al.* , 1996
	Mercenaria mercenaria	DG	Tampa Bay, Florida	PAHs, PCBs, DDTs and Me	Transplantation	—	Nasci *et al.* , 1999
	Mytilus galloprovincialis	DG	Ebro Delta	PAHs	Seasonal variation	↑	Solé *et al.* , 1995
		DG		PCBs		↑	
		DG		DDTs		↑	
		DG	Venice Lagoon	PAHs, PCBs, DDTs	Spatial variation	—	Livingstone *et al.* , 1995
		DG	Bay of Biscay	PAHs, PCBs	Spatial and seasonal variation	—	Orbea *et al.* , 2002
	Perna viridis	Gills	Hong Kong	PAHs	Transplantation	—	Cheung *et al.* , 2001
		DG	Hong Kong	PAHs	Transplantation	↑	
	Saccostrea cucullata	DG	Hooghly Estuary, India	PAHs	Spatial and seasonal variation	↑	Niyogi *et al.* , 2001
CAT	*Mytilus galloprovincialis*	DG	Venice Lagoon	PAHs, PCBs, DDTs	Spatial variation	—	Livingstone *et al.* , 1995
		DG	Bay of Biscay	PAHs, PCBs	Spatial and seasonal variation	—	Orbea *et al.* , 2002
	Crassostrea sp.	DG	Bay of Biscay	PAHs, PCBs	Spatial and seasonal variation	—	
	Perna viridis	Gills	Hong Kong	PAHs	Transplantation	↑	Cheung *et al.* , 2001
		DG	Hong Kong	PAHs	Transplantation	—	
	Mercenaria mercenaria	DG	Tampa Bay, Florida	PAHs, PCBs, DDTs and Me	Transplantation	↑	Nasci *et al.* , 1999
	Tapes phillipinarum	DG	Venice Lagoon	PAHs, PCBs, DDTs	Transplantation	—	Nasci *et al.* , 2000
	Saccostrea cucullata	DG	Hooghly Estuary, India	PAHs	Spatial and seasonal variation	↑	Niyogi *et al.* , 2001
	Mytilus galloprovincialis	DG	—	BaP (50 mg/kg dry weight mussel)	Laboratory exposure	↑	Akcha *et al.* , 2000
T GPx	*Perna viridis*	Gills	Hong Kong	PAHs	Transplantation	↑	Cheung *et al.* , 2001
		DG	Hong Kong	PAHs	Transplantation	—	Cheung *et al.* , 2001
	Unio tumidus	DG	French Rivers	PAHs, PCBs, DDTs and Me	Transplantation	↓	Cossu *et al.* , 2000
	Saccostrea cucullata	DG	Hooghly Estuary, India	PAHs	Spatial and seasonal variation	—	Niyogi *et al.* , 2001
Se GPx	*Tapes phillipinarum*	DG	Venice Lagoon	PAHs, PCBs, DDTs	Transplantation	↓	Nasci *et al.* , 2000
	Mytilus galloprovincialis	DG	Ebro Delta	PAHs	Seasonal variation	—	Solé *et al.* , 1995
		DG		Lindane		↑	
		DG	Bay of Biscay	PAHs, PCBs	Spatial and seasonal variation	—	Orbea *et al.* , 2002
	Unio tumidus	DG	French Rivers	PAHs, PCBs, DDTs and Me	Transplantation	↓	Cossu *et al.* , 2000
	Crassostrea sp.	DG	Bay of Biscay	PAHs, PCBs	Spatial and seasonal variation	—	
LPO	*Perna viridis*	Gills	Hong Kong	PAHs	Transplantation	—	Cheung *et al.* , 2001
		DG	Hong Kong	PAHs	Transplantation	—	
	Mytilus edulis	DG	Galician Coast	oil contamination	Seasonal variation	↑	Porte *et al.* , 1996
	Unio tumidus	Whole	French Rivers	PAHs, PCBs, DDTs and Me	Transplantation	↑	Cossu *et al.* , 2000
	Saccostrea cucullata	DG	Hooghly Estuary, India	PAHs	Spatial and seasonal variation	—	Niyogi *et al.* , 2001
BPH	*Mercenaria mercenaria*	DG	Tampa Bay, Florida	PAHs, PCBs, DDTs and Me	Transplantation	↑	Nasci *et al.* , 1999
	Tapes phillipinarum	DG	Venice Lagoon	PAHs, PCBs, DDTs	Transplantation	—	Nasci *et al.* , 2000
	Mytilus galloprovincialis	DG	—	BaP (50 mg/kg dry weight mussel)	Laboratory exposure	↑	Akcha *et al.* , 2000
		DG	—	3-methylcolanthrene (1 mg/mussel)	Laboratory exposure	↑	Michel *et al.* , 1994
		DG	Mediterranean Sea	PAHs	Spatial variation	↑	Michel *et al.* , 1994
		DG	South western France	oil contamination	Spatial variation	↑	Michel *et al.* , 1994
		DG	Galician Coast	oil contamination	Spatial variation	—	Porte *et al.* , 2001
	Mytilus edulis	DG	South Wales, UK	oil contamination	Spatial variation	↑	Michel *et al.* , 1994
GST	*Ruditapes decussatus*	Whole	—	BaP (1.5 - 3 µg/L)	Laboratory exposure	↑	Hoarau *et al.* , 2001
	Tapes decussata	DG	Galician Coast	oil contamination	Seasonal variation	↑	Porte *et al.* , 1996
	Mytilus galloprovincialis	DG	Venice Lagoon	PAHs, PCBs, DDTs	Spatial variation	—	Livingstone *et al.* , 1995
		Gills	—	BaP (50 mg/kg dry weight mussel)	Laboratory exposure	↓	Akcha *et al.* , 2000
		DG	—	BaP (50 mg/kg dry weight mussel)	Laboratory exposure	—	
	Perna viridis	DG	Hong Kong	PAHs	Transplantation	↑	Cheung *et al.* , 2001
		Gills	Hong Kong	PAHs	Transplantation	↑	
	Crassostrea sp.	DG	Bay of Biscay	PAHs, PCBs	Spatial and seasonal variation	—	Orbea *et al.* , 2002

DG – digestive gland; ↑ - increase; ↓ - decrease; — - no effect.

Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) whose variation have been demonstrated in laboratory and field studies conducted with bivalve molluscs are considered as general biomarkers of PAHs mediated oxidative stress (Porte *et al.*, 1991; Solé *et al.*, 1994; Solé *et al.*, 1995; Livingstone, 2001). However, these responses were generally transient and variable for different species, enzymes and single or mixed contaminants (Livingstone, 2001). The activity of Se dependent GPx in the clams *T. phillipinarum* transplanted to PAHs, PCBs and DDTs contaminated sites in the Venice Lagoon decreased. However, SOD and CAT activities were unaltered (Nasci *et al.*, 2000) while in oil contaminated spots in the Galician Coast, SOD activity increased (Porte *et al.*, 1996). Clams *M. mercenaria* transplanted to PAHs, PCBs, DDTs and metal contaminated sites in Tampa Bay (Florida) did not show any alteration in SOD activity while CAT activity increased (Nasci *et al.*, 1999). Similarly, in *M. galloprovincialis* from PAHs and PCBs contaminated sites in the Bay of Biscay no effect in antioxidant enzymes activities was observed (SOD, CAT and Se GPx) (Orbea *et al.*, 2002). However, in mussels *P. viridis* transplanted to a PAHs contaminated site in Hong Kong the activities of SOD in the digestive gland and of CAT and T GPx in the gills increased (Cheung *et al.*, 2001).

Impairment of these antioxidant defences and the reactive oxygen species produced in PAHs metabolism may lead to oxidative stress, resulting in peroxidation of membrane lipids. Thus, lipid peroxidation may be considered a biomarker of oxidative damage in organisms exposed to PAHs (Livingstone *et al.*, 2000). This was demonstrated in the digestive gland of mussels *M. edulis* from oil contaminated sites in the Galician Coast (Porte *et al.*, 1996) and also in the whole tissues of mussels *Unio tumidus* transplanted to PAHs, PCBs, DDTs and metals contaminated sites in France (Cossu *et al.*, 2000). Conversely, no lipid peroxidation was observed in neither the gills nor the digestive gland of mussels *P. viridis* transplanted to a PAHs contaminated site in Hong Kong (Cheung *et al.*, 2001).

Most of the mentioned biomarkers have been followed in laboratory studies under controlled conditions. Although these studies may help to establish cause/effect relationships, they fail to detect the influence of a number of biotic and abiotic factors (Hyne & Maher, 2003). Previous studies have demonstrated that biomarkers may be influenced by seasonal factors, including temperature, gametogenesis, salinity, diet and sunlight exposure (Cotelle & Férard, 1999; Livingstone et al., 1990; Sheehan & Power, 1999; Sleiderink et al., 1995). Thus, biomarker induction or inhibition may be related to seasonal effects, whether exogenous or endogenous, as well as contaminant exposure, or interactions of both. Hence, it is important to characterise biomarker seasonal variation to assist data interpretation (Shaw et al., 2004).

1.5. THE RIA FORMOSA LAGOON

The Ria Formosa is a coastal lagoon situated in the South Portuguese coast. It is about 55 km long and its area reaches 160 km^2. It comprises mainland, back-barrier lagoons, inlet deltas, barrier islands, barrier platforms and shoreface and is permanently connected to the sea through several narrow inlets. This system of barrier islands is delimited in the west by the Ancão Peninsula (followed from west to east by the islands of Barreta, Culatra, Armona, Tavira and Cabanas) and to the east by Cacela Peninsula (Bernardo *et al.*, 2002). These islands are inhabited mainly by fishermen (Bernardo *et al.*, 2002; Newton & Mudge, 2005). Connection with the sea is made through six inlets, which divide the five barrier islands. The main inlet of the system is the Farol-Olhão inlet, which has been artificially consolidated (Newton & Mudge, 2003).

The Ria Formosa is a shallow lagoon with a mean depth of approximately 3.5 m. Its tidal amplitude varies from a maximum of 3.5 m at spring tide to 0.5 m at neap tide. The intertidal area exposed at low tide may reach 50 km^2 (ca. 30% of the total lagoon area). There is a rather intense exchange of 50 – 75 % of the water mass during each tide (Newton & Mudge, 2003).

There are five rivers discharging to the Ria Formosa, however four of these are seasonal. The River Gilão (Tavira) is the only permanent freshwater input to the lagoon. Rainfall is occasional, occurring mainly in the winter (http://snirh.inag.pt; Newton & Mudge, 2003).

Salinity is in the range of 35.5 to 36.9 ‰ all year round (Falcão *et al.*, 1985). However, despite the large tidal exchange of water, the inner channels of the Ria Formosa are brackish in winter and hypersaline in summer (Newton & Mudge, 2003). Water temperature varies from 12-13ºC in winter, to 27-28ºC in summer. However, in summer and during the ebb tides, water temperature can rise to approximately 30ºC in the more inland and shallower zones of the lagoon (Chícharo & Chícharo, 2001a).

The Ria Formosa sediments are in general of sand and silt nature. The barrier islands are mainly of coarse sandy sediments, but the shores of the inner channels are mainly of silt muddy sediments (Bettencourt, 1994).

Benthic macrophytes, such as the salt-marsh species *Spartina maritima*, the seagrass species *Zostera noltii* and *Cymodocea nodosa*, and green mat-forming macroalgae (Ulvales) are considered the main primary producers in this lagoon (Sprung, 1994).

It is a Natural Park but the Ria Formosa also plays an important role in the region economy. Beyond the tourist use, the system is also the support of other economic activities like seafood culture and port facilities (Silva *et al.*, 2002). The Ria Formosa lagoon plays also an important ecological role as a breeding ground and nursery to several species (Pereira & Machado, 1987). There is also a long tradition of bivalve harvesting, especially of *Ruditapes decussatus* Linnaeus 1758, whose growth-banks occupy about 20% of the lagoon intertidal area, and represents 90% of total portuguese seafood production (Falcão & Vale, 1990; Chícharo & Chícharo, 2001b). Although clam production has decreased significantly in the last years (Chícharo & Chícharo, 2001a; Leite *et al.*, 2004), this economic activity still involves directly or indirectly over 10,000 people in this region (Leite *et al.*, 2004).

Contamination of the lagoonal system occurs mainly through sewage discharges (Lima & Vale, 1980; Pereira & Machado, 1987), industrial effluents, agriculture runoff (Gil & Vale, 1999), mariculture and port activities (Coelho *et al.*, 2002c) and several studies regarding the levels of inorganic and organic contaminants in sediments and in organisms are in Tables 1.10 and 1.11.

Metal concentrations in sediments ranged from 0.04 to 6.3 μg g^{-1} d.w. for cadmium, 0.98 to 188.3 μg g^{-1} d.w. for zinc, 4.2 to 91 μg g^{-1} d.w. for lead and 0.7 to 55 μg g^{-1} d.w. for copper (Bebianno, 1995; Padinha *et al.*, 2000; Caetano *et al.*, 2002). Sterol and fatty acid concentrations in the sediments ranged from 0.1 to 1654 μg g^{-1} d.w. and from 0.3 to 1700 μg g^{-1} d.w., respectively and specific ratios indicated that part of the organic matter in the settling sediments of the Ria Formosa lagoon comes from sewage sources (Mudge & Bebianno, 1997; Mudge *et al.*, 1998; Mudge *et al.*, 1999; Mudge & Duce, 2005). Tributyltin (TBT) ranged from 3 to 30 ng g^{-1} d.w. in the Ria Formosa sediments (Gibbs *et al.*, 1997; Coelho *et al.*, 2002a; Díez *et al.*, 2005). The other analysed compounds, namely dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs) (Gil & Vale, 1999; Ferreira & Vale, 1995; Castro & Vale, 1995; Ferreira & Vale, 1998; Barreira *et al.*, 2005) were present in the sediments at very low concentrations. Concerning the PAHs, only one study exist in the literature, in which PAH concentrations were determined in the sediments (Table 1.10) and settled particles (Veriato *et al.*, 1998) expressed as chrysene equivalents (Table 1.10) and no information on individual PAHs was reported. Thus the information on the Ria Formosa sediment PAHs remains limited.

In terms of contaminants in organisms, the bivalves *M. galloprovincialis*, *R. decussatus* and *C. angulata* are among the most studied, mainly due to their abundance in the Ria Formosa and also because they are biomonitors, important edible species and may pose a potential risk for human consumption (Table 1.11). In these bivalves, metal concentrations were in the same order of magnitude as other bivalve species from Mediterranean Coast (Bebianno & Serafim, 2003).

PCB and DDT levels were low and also similar to those found in other Mediterranean species (Solé *et al.*, 1994; Orbea *et al.*, 2002). TBT levels, however, were above the Environmental Quality Standards (Coelho *et al.*, 2002c).

Table 1.10 – Inorganic and organic contaminants in the sediments of the Ria Formosa lagoon.

Contaminants	Concentration Range	Period	Reference
Inorganic			
Cd	0.13 – 4.65 *	-	Bebianno, 1995
	0.04 – 0.52 *	1992/3	Caetano *et al.* , 2002
	4.0 - 6.3 *	1996	Padinha *et al.* , 2000
Zn	0.98 – 188.3 *	-	Bebianno, 1995
	78 - 155 *	1996	Padinha *et al.* , 2000
Pb	4.2 – 42.2 *	-	Bebianno, 1995
	40 - 91 *	1996	Padinha *et al.* , 2000
Cu	0.7 – 23.7 *	-	Bebianno, 1995
	10 – 43 *	1992/3	Caetano *et al.* , 2002
	28 - 55 *	1996	Padinha *et al.* , 2000
Organic			
Sterols	0.4 - 117.4 *	1994	Mudge & Bebianno, 1997
	0.1 – 27.8 *	1995	Mudge *et al.* , 1999
	0.11 - 1654 *	2000	Mudge & Duce, 2005
Fatty Acids	0.3 – 1700 *	1995	Mudge *et al.* , 1998
PCB	0.1 – 1.9 **	1989	Castro & Vale, 1995
	5 – 12 **	1990	Ferreira & Vale, 1995
	0.10 – 2.10 **	1996/7	Barreira *et al.* , 2005
DDT	0.16 – 1.12 **	1989	Gil & Vale, 1999
	2 – 7 **	1990	Ferreira & Vale, 1995
PAH	0.4 – 6.4 ***	1996/97	Veriato *et al.* , 1998
TBT	5-30 **	1992/3	Coelho *et al.* , 2002c
	3-5 **	1999/2000	Díez *et al.* , 2005

* μg g^{-1} d.w.; ** ng g^{-1} d.w.; *** μg chrysene eq g^{-1} d.w.

Table 1.11 – Inorganic and organic contaminants in bivalves from the Ria Formosa lagoon.

Contaminants	Species	Tissue	Concentration Range	Period	Reference
Inorganic					
Cd	*Mytilus galloprovincialis*	Whole Soft Tissues	2.1 – 2.7 [b]	1994	Bebianno & Machado, 1997
			0.36 - 0.69 [b]	2001	Morgado & Bebianno, 2005
	Ruditapes decussatus	Gills	3.65 – 19.27 [a]	1994-95	Bebianno & Serafim, 2003
		Digestive Gland	5.16 – 29.76 [a]		
		Remaining Tissues	1.38 – 9.69 [a]		
Zn	*Mytilus galloprovincialis*	Whole Soft Tissues	200 – 361 [b]	1994	Bebianno & Machado, 1997
			156 - 321[b]	2001	Morgado & Bebianno, 2005
	Ruditapes decussatus	Gills	1255 – 2074 [a]	1994-95	Bebianno & Serafim, 2003
		Digestive Gland	1083 – 1645 [a]		
		Remaining Tissues	738 – 1114 [a]		
Cu	*Mytilus galloprovincialis*	Whole Soft Tissues	5.7 – 6.1 [b]	1994	Bebianno & Machado, 1997
			4.57 - 6.35 [b]	2001	Morgado & Bebianno, 2005
	Ruditapes decussatus	Gills	96.3 – 241.0 [a]	1994-95	Bebianno & Serafim, 2003
		Digestive Gland	131.3 – 205.7 [a]		
		Remaining Tissues	68.0 – 166.7 [a]		
Ag	*Mytilus galloprovincialis*	Whole Soft Tissues	0.0036 - 0.0082 [b]	2001	Morgado & Bebianno, 2005
Ni			0.22 - 0.61 [b]		
Cr			0.46 - 1.01 [b]		
Pb			0.84 - 2.89 [b]		
Organic					
TBT	*Ruditapes decussatus*	Whole Soft Tissues	< 0.15 [c]	-	Coelho *et al.* , 2002a
PCB	*Crassostrea angulata*		0.2 – 1.0 [d]	1990	Ferreira & Vale, 1995
			2 – 40 [e]	-	Ferreira & Vale, 1998
	Ruditapes decussatus		9 – 52 [e]	-	
DDT	*Crassostrea angulata*		0.3 – 2.1 [d]	1990	Ferreira & Vale, 1995

[a] nmol g^{-1}; [b] µg g^{-1}; [c] µg Sn g^{-1}; [d] µg g^{-1} lipids; [e] ng g^{-1}.

1.6. THE CLAM *RUDITAPES DECUSSATUS*

The clam *R. decussatus*, also known as the grooved carpet shell clam, is a suspension dwelling feeder bivalve (Bebianno *et al.*, 2004). It is amply distributed through the Atlantic coast from England to Africa, in the south of the Mediterranean (Bebianno *et al.*, 2004) and West Africa. In Portugal is found in several locations, in the Ria de Aveiro, Tejo estuary, Tróia, Sado estuary, Ria de Alvor and Ria Formosa (Leite *et al.*, 2004). However, the Ria Formosa represents 90% of the national farming of these clams (Chícharo & Chícharo, 2001a). This species is generally found in sandy or muddy grounds, where it lives in tidal flats, buried in the sediments in relatively undisturbed areas. Younger individuals are found at the surface sediments; however older individuals may be found buried up to 10 cm into the sediment (Chícharo & Chícharo, 2001b).

Bivalve production in the Ria Formosa has decreased significantly in the last years (from 3-4 kg m^{-2} to 500 g m^{-2}) and mortality has increased concurrently to 50%, although episodic events leading to 100% mortality of this species have occurred in some areas (Ferreira *et al.*, 1989; Mudge & Bebianno, 1997; Chícharo & Chícharo, 2001a). Although a final link remains to be established, this mortality has been associated to environmental pollution (Bebianno, 1995; Mudge & Bebianno, 1997; Bebianno & Serafim, 2003; Newton *et al.*, 2003) and parasitism (Leite *et al.*, 2004; Elandalloussi *et al.*, 2005).

R. decussatus has two siphons, one of which is the inhalant siphon that allows filtration of water and suspended particles and the other used for water and waste extraction (Bebianno *et al.*, 2004). In such a dynamic system, as the Ria Formosa, ressuspension of sediments is a common feature and the ability of clams to filter and select appropriate food particles is determinant on their performance and growth (Sobral & Widdows, 2000). Due to

its feeding behaviour and low metabolism (Porte et al., 2000; Solé et al., 2000), *R. decussatus* accumulates, in its tissues a large number of inorganic (metals) and organic contaminants such as TBT, PCBs and PAHs (see Tables 1.4 and 1.11), reflecting the gradient of contamination in the environment (Bebianno *et al.*, 2004). For this reason, this species has already been used as a bioindicator species of inorganic (Moraga et al., 2002; Bebianno & Serafim, 2003; Beiras & Albentosa, 2004; Smaoui-Damak *et al.*, 2004) and organic contaminants (Solé et al., 1994; Porte et al., 1996; Ferreira & Vale, 1998; Porte et al., 1998; Solé, 2000b; Solé et al., 2000; Dellali *et al.*, 2001; Coelho *et al.*, 2002c; Binelli & Provini, 2003).

R. decussatus was recently the object of a review on its use as a bioindicator species for both inorganic and organic contamination, in sites where mussels are not easily obtained (Bebianno *et al.*, 2004). In this review, several biomarkers as metallothioneins (MT), different antioxidant enzymes (SOD, CAT and total and selenium dependent GPx), glutathione S-transferase (GST) and acetylcholinesterase (AChE) or products resulting from lipid peroxidation (LPO), were highlighted as relevant biomarkers in *R. decussatus* gills or digestive gland. Most of the highlighted relationships were, however, with metals emphasizing the need of further studies on the relationship between biochemical biomarkers and organic contaminants in *R. decussatus*.

In summary, this species ability to accumulate organic contaminants, such as the PAHs, its abundance in the Ria Formosa, and its importance in terms of human consumption make of *R. decussatus* a very useful biological model for this study.

1.7. OBJECTIVE

The main objective of this thesis was to improve the knowledge on accumulation mechanisms of polycyclic aromatic hydrocarbons in the clams *R. decussatus* from the Ria Formosa lagoon (Portugal) and their relationship with oxidative stress parameters.

Polycyclic aromatic hydrocarbons (PAHs) constitute one of the most important classes of environmental contaminants, due to their widespread distribution and toxicity (Solé, 2000a). In the first chapter of this dissertation, a review of the PAH physico/chemical properties, as well as their formation and sources to the aquatic environment is presented. This chapter focuses also on the PAH concentrations and fate in seawater and sediments, two of the major compartments of the marine environment. PAHs accumulation and transformation by marine organisms was also described focusing on the mechanisms of PAHs metabolism by marine molluscs. The mechanisms leading to the formation of reactive oxygen species (ROS) and therefore to the induction of antioxidant defences were also highlighted. In particular, antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidases) were characterized, including their mechanism of action, localization within the cell compartment and tissue, existence of different isoforms, etc. Some attention was also given to oxidative damage occurring in cells due to ROS production, in particular the peroxidation of membrane lipids (LPO). Furthermore, the role of these antioxidant enzymes and LPO as biomarkers of PAHs on different bivalve species was considered including a bibliographic review of the more relevant studies in this area. Additionally, a description of the study area and of the selected species was included.

In order to relate the oxidative stress markers with the PAHs, it was necessary to know PAH concentrations in the Ria Formosa lagoon: their spatial and seasonal variation, focusing not only on their levels present in the sediments or accumulated in organisms but also their signatures, which may help to clarify PAH origins in this lagoon system. The sediments were

selected since they act as a natural sink of organic matter, and therefore of most of the organic contaminants (see section 1.1.4). Therefore, Chapter 2 focuses on the spatial and seasonal variation of PAH concentrations in the sediments.

Bivalve molluscs are widely used as bioindicators of environmental contamination due to their ability to accumulate inorganic and organic contaminants, providing information on spatial and temporal contamination trends (Solé *et al.*, 2000). Clams *R. decussatus* are suspension feeder bivalves of high economic value that live in the sediments. These clams are extensively cultured and widespread in the Ria Formosa lagoon. Therefore, the accumulation of toxic compounds in this clam edible part is highly relevant for human health (Gilroy, 2000; Binelli & Provini, 2003). Thus, in Chapter 3, PAH concentrations in the whole soft tissues of *R. decussatus* from several sites of the Ria Formosa lagoon were studied, as well as their spatial and seasonal variation. The relationship between PAH concentrations in the clam tissues and those found in the sediments (Chapter 2) were assessed.

Following exposure to PAHs, organisms tend to metabolise and eliminate them as metabolites, minimizing cellular damage. Some of these metabolites are capable of undergoing redox cycling originating oxyradicals which often induce antioxidant defence systems (Livingstone *et al.*, 1990; Livingstone *et al.*, 1992). These oxidative stress related parameters were studied in bivalve species and related to exposure to inorganic and organic xenobiotics (Solé *et al.*, 1994). However, there are still some reservations on their application as biomarkers of PAHs mediated oxidative stress in field studies (De Luca-Abbott *et al.*, 2005). Therefore, Chapter 4 describes the oxidative stress related markers in the digestive gland of *R. decussatus* and their spatial and seasonal variations. The activities of four antioxidant enzymes, considered general biomarkers of environmental contamination, including PAHs, were studied. These were superoxide dismutase (SOD), catalase (CAT) and total and selenium-dependent glutathione peroxidases (T GPx and Se GPx). The digestive

gland was selected as the main tissue responsible for the biotransformation of lipophilic organic xenobiotics. Lipid peroxidation products (LPO) were also measured as markers of oxidative damage. Antioxidant enzymatic activities and LPO levels were then related to PAH concentrations in *R. decussatus* whole soft tissues.

The results described in Chapter 4, illustrated the seasonal behaviour of antioxidant enzymes in *R. decussatus* in the Ria Formosa lagoon. Usually, laboratory experiments required to provide evidence of unambiguous relationships between contaminants and biomarkers failed to reproduce real environmental conditions (Viarengo *et al.*, 1991; Viarengo *et al.*, 1995; Pannunzio & Storey, 1998; Ross *et al.*, 2001; Niyogi *et al.*, 2001b; Abele *et al.*, 2002; Vidal *et al.*, 2002a). In Chapter 5, an active biomonitoring experiment was designed in which clams were transplanted between two sites with different background PAH concentrations (identified in Chapters 2 and 3). Bioactive monitoring associated with the measure of biochemical parameters allows for the establishment of causal relationships between the accumulation of contaminants and their effects in real environmental conditions (Nasci *et al.*, 1999; Roméo *et al.*, 2003b). In this experiment PAHs bioaccumulation and elimination by *R. decussatus* were studied, along with their biological effects, including antioxidant enzymes, lipid peroxidation and the activity of two enzymes from phase I and phase II of the metabolism of lipophilic xenobiotics in order to understand PAHs metabolisation in *R. decussatus*. These two enzymes were benzo[*a*]pyrene hydroxylase (BPH) and glutathione S-transferase (GST) (see Chapter 1). Both the enzymatic activities and LPO levels were measured in the gills in addition to the digestive gland. Although, as mentioned before, the digestive gland is considered the main organ for PAHs metabolisation (Livingstone *et al.*, 1985; Solé *et al.*, 1994) the gills have an important role in the uptake and excretion of xenobiotic compounds in this species (Andersson & Förlin, 1992; Fossi *et al.*,

2000). Therefore, some authors highlighted that the gills may be more sensitive to PAHs exposure (Cheung *et al.*, 2001; Hoarau *et al.*, 2001).

Finally, in Chapter 6, a general discussion is presented, focusing on the major conclusions, discussing the PAH concentrations and possible sources to the clams in the Ria Formosa lagoon and the relationship between antioxidant enzymes, BPH, GST and LPO and PAH concentrations in *R. decussatus.*

2. POLYCYCLIC AROMATIC HYDROCARBONS IN THE SEDIMENTS

2.1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a widespread class of environmental contaminants (Notar *et al.*, 2001). They are generally the result of thermal decomposition of organic molecules and the subsequent recombination of the newly formed organic fractions (pyrolysis). PAHs are known human carcinogens (Anderson *et al.*, 2002; Schneider *et al.*, 2002) and their toxicity to aquatic life has also been amply documented (Anderson, 1985; Akcha *et al.*, 2000; Binelli & Provini, 2003), for this reason they are considered priority pollutants (Solé, 2000a).

PAHs may have both natural (*e.g.* forest fires, natural oil seeps, diagenesis of sedimented organic matter) and anthropogenic origins (*e.g.* combustion of fossil fuels, accidental oil spills, waste incineration, coke and asphalt production), however the major fraction of PAHs in the environment are due to mankind actions (Bi *et al.*, 2002; Gabos *et al.*, 2001; Johansson & von Bavel, 2003; Margarido, 1999; Simcik *et al.*, 1999; Slater *et al.*, 2002; Porte *et al.*, 2000). Depending on their mechanism of formation, PAHs may be designated as pyrolytic (produced during incomplete combustion or pyrolisis of organic matter at high temperatures), petrogenic (produced by rearrangement and transformation of biogenic organic materials at moderate temperatures – fossil fuels), diagenic (originated in the transformation of certain organic compounds in soils and sediments) or biogenic (synthethised by organisms) (Neff, 2002).

PAHs may enter the aquatic environment *via* atmospheric fallout (Dickhut & Gustafson, 1995; Simcik *et al.*, 1999; Dickhut *et al.*, 2000; Arzayus *et al.*, 2001; Gabos *et al.*, 2001; Golomb *et al.*, 2001), urban run-off (McCready *et al.*, 2000; Friedman *et al.*, 2000), spillage of petroleum (Page *et al.*, 1999; Porte *et al.*, 2000; Pastor *et al.*, 2001; Barakat *et al.*, 2002) and municipal and industrial effluents (Marttinen *et al.*, 2003). PAHs in the environment rapidly adsorb to particles

and settle in sediments due to their hydrophobic nature (Borglin *et al.*, 1996; Dabestani & Ivanov, 1999; Kirso *et al.*, 2001). The PAHs high octanol/water partitioning coefficient (K_{ow}) and greater persistence of sedimentary PAHs compared to PAHs in solution, make sediments useful in identifying inputs to the environment (Benlahcen *et al.*, 1997; Baumard *et al.*, 1998a; Guinan *et al.*, 2001; Notar *et al.*, 2001).

Each individual PAH source is characterized by a specific molecular pattern, allowing the source of these compounds to be established (Burns *et al.*, 1997; Baumard *et al.*, 1999a; Savinov *et al.*, 2000). In this way, PAH ratio profiles have been used as a means of differentiating between pyrolytic and petrogenic contamination origins (Yang, 2000; Guinan *et al.*, 2001). Additionally, recently developed multivariate statistical techniques such as PLS analysis have been used in the apportionment of the PAH contamination sources (Mudge, 2002). Briefly, this technique uses PAH data from pure source samples or single-source rich environmental samples as fingerprints, which are projected on the sample datasets thereby quantifying the amount of variance that can be explained or predicted by each source (Mudge, 2002).

The Ria Formosa lagoon is a coastal lagoon on the Southern coast of Portugal (see section 1.5 for description). The water quality of this lagoon has deteriorated over recent years reflecting the intense economic pressure around the lagoon. Major inputs include domestic and industrial sewage discharges, agricultural and road run-off, ports, marinas and aquaculture (Lima & Vale, 1980; Pereira & Machado, 1987; Bebianno, 1995; Gil & Vale, 1999; Coelho *et al.*, 2002c; Barreira *et al.*, 2005). The sediment concentrations of anthropogenic organic compounds, namely polychlorinated biphenyls (PCBs), dichloro-diphenyl-trichloroethane (DDT), tributyltin (TBT), sterols and fatty acids are known (see section 1.5 for a detailed review), but the knowledge on PAH concentrations in the Ria Formosa lagoon is limited (Veriato *et al.*, 1998).

A detailed study was conducted to investigate the composition, distribution and sources of PAHs in the Ria Formosa lagoon. Eight sites were selected according to their proximity to urban areas, sediment nature and water exchange. Sixteen parent PAHs, which are in the EPA priority list: naphthalene (N), acenaphthylene (Ace), acenaphthene (Ac), fluorene (F), phenanthrene (P), anthracene (A), fluoranthene (Fluo), pyrene (Py), benzo[*a*]anthracene (BaA), chrysene (Ch), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), benzo[*a*]pyrene (BaP), dibenzo[*a,h*] anthracene (DBA), benzo[*g,h,i*]perylene (Bper) and indene[123-*cd*]pyrene (IP) were analysed in surface sediments.

Besides spatial variations in sediment PAH concentrations, seasonal variation may occur due to changes in PAH sources to the environment. Higher PAH concentrations exist in sediments from the Western Baltic Sea in the summer, related to the increase in maritime traffic (Baumard *et al.*, 1999a). Seasonal variation also exists in PAH concentrations in water bodies of the Baltic Sea, with higher concentrations in the winter reflecting the increase in combustion sources in this season (Witt, 2002).

Therefore, sampling was performed at different times to study seasonal variation in the PAH levels and sources in the Ria Formosa and improve the knowledge of these compounds in this area.

2.2. MATERIALS AND METHODS

2.2.1. Sampling

Sediment samples were collected at 8 different sites of the Ria Formosa Lagoon (Figure 2.1).

Sampling sites vary in (a) sediment grain size: sites 1, 2, 3 and 4 were sandy sediments and sites 5, 6, 7 and 8 were muddy sediments; (b) water exchange: sites 5, 6 and 8 had a

restricted water exchange compared to the others as they were located in the inner parts of the Ria Formosa; and (c) geography: site 6 was close to urban areas, sites 1 and 4 were located near the outlets to the ocean and site 7 was close to both.

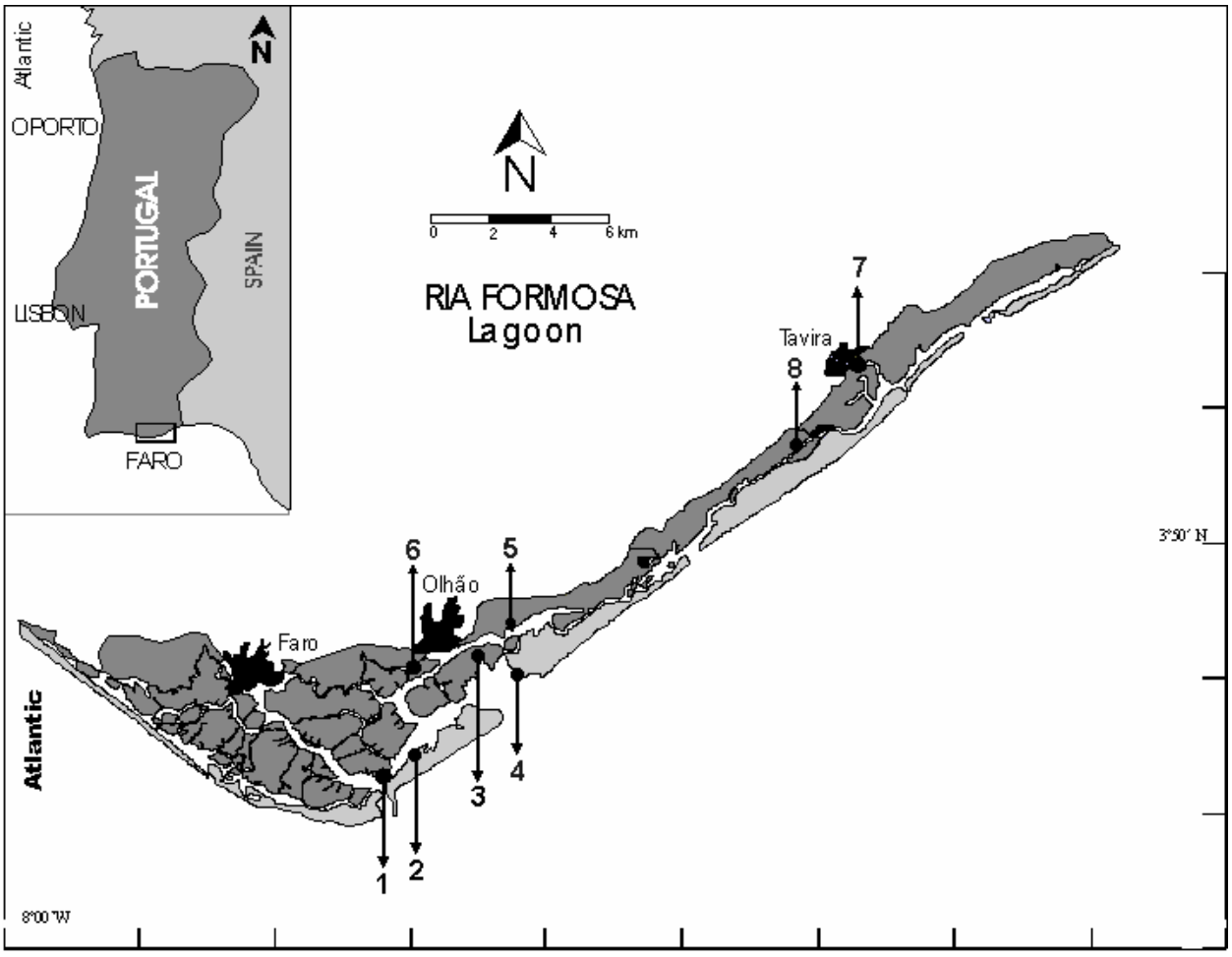


Figure 2.1 – Sample locations in the Ria Formosa.
Legend: 1 – Farol; 2 – Culatra; 3 – Lameirão; 4 – Armona; 5 – Marim; 6 – Olhão; 7 – Quatro Águas; 8 – Torre d'Aires.

Sampling took place between January and August 1995 at low tide. All eight sites were sampled in January, May and August. In March, June and July, sampling was reduced to sites 1, 4, 6 and 7 due to logistic reasons. Altogether, 36 samples were collected and analysed in triplicate. Surface sediments (top 5 cm) were collected with pre-cleaned glass jars with a plastic

top enclosed by an aluminium foil. Samples were kept at 4ºC prior to their arrival at the laboratory where they were kept at –20ºC until analysis.

2.2.2. PAHs sediment analysis

The sixteen EPA (Environmental Protection Agency) recommended PAHs were analysed in the sediment samples in triplicate. About 40 g of each field-moist sediment sample was refluxed in 50 ml of 6% KOH methanol solution for 4 h. After cooling, samples were centrifuged to separate the supernatant from the sediment and the extracts were decanted into a separating funnel. The aromatic hydrocarbons were then extracted twice with 50 ml of hexane. Both solvent fractions were combined and their volume reduced in a rotary evaporator. The PAHs were further purified on an activated silica/alumina column. The aliphatic fraction was removed with 40 ml of hexane and the aromatic fraction was eluted with 25 ml of hexane/dichloromethane (9:1) and 25 ml of H/DCM (4:1). The aromatic fractions were combined and taken to complete dryness by a combination of rotary evaporation and a gentle nitrogen stream. The PAH separation and quantification was performed by HPLC-UV (Thermo Separations Products). For the HPLC procedure, samples were resuspended in 300 µl of acetonitrile and 20 µl of sample was injected on a C-18 reversed phase column (LiChrospher PAH, Merck ref. 50156), using a rheodyne type injector. The PAH separation was achieved by an acetonitrile/water gradient varying in time. The gradient of elution was: 50% acetonitrile and 50% water from 0 to 15 min, changing to 100% acetonitrile from 15 to 20 min and maintaining this composition until 35 min (Figure 2.2).

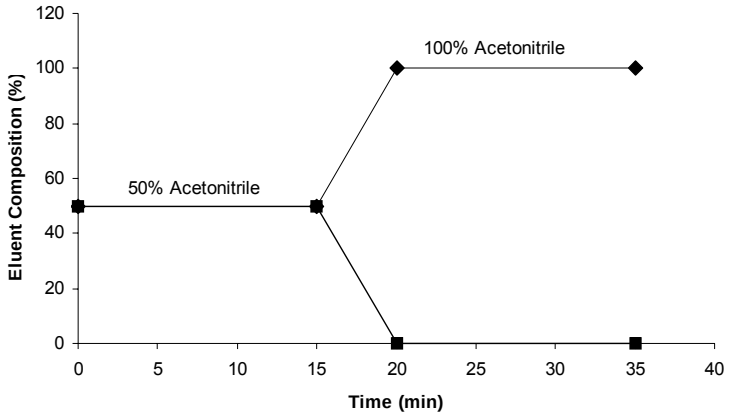


Figure 2.2 – Elution gradient.

A good resolution for all peaks was achieved with the exception of acenaphthene and fluorene that frequently co-elute (Figure 2.3).

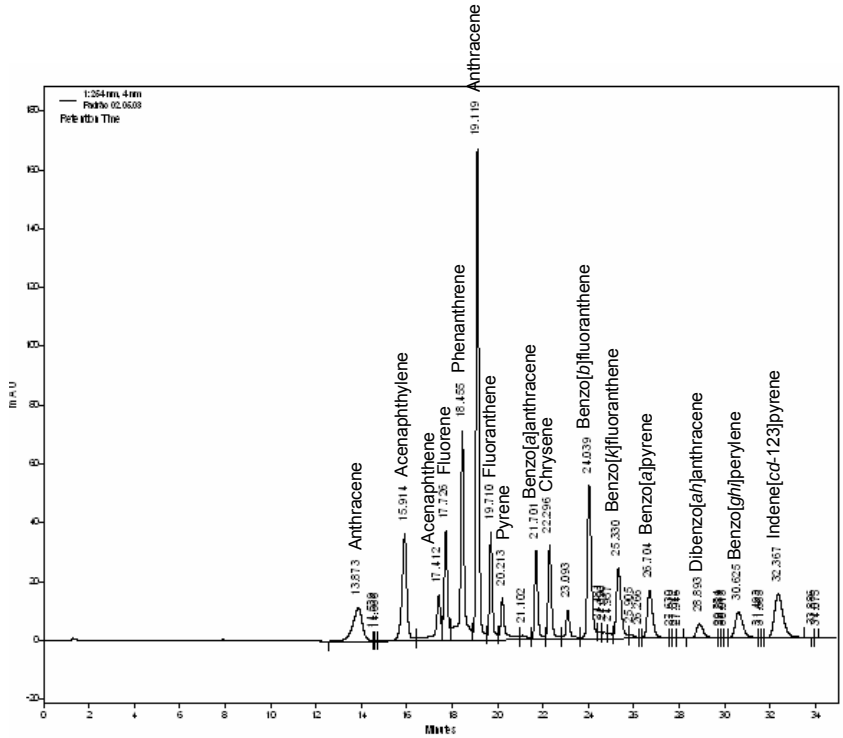


Figure 2.3 – Chromatogram of a composed standard of the 16 PAHs.

In the samples, peak identification was assessed by comparison of retention times based on phenanthrene (Figure 2.4) and confirmed by spectra comparison between 200 and 450 nm with the pure compounds. Phenanthrene was chosen as a reference peak since it was easily identified and was present in all samples.

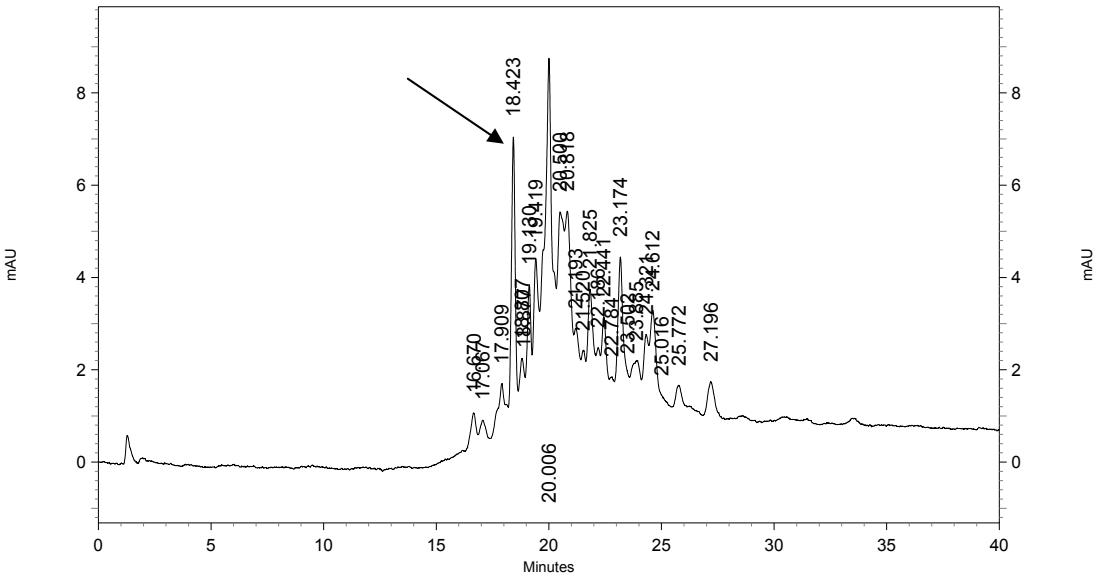


Figure 2.4 – Chromatogram of a representative sediment sample. Phenanthrene peak is marked.

Quantification was performed using the peak areas at 254 nm with calibration curves constructed for each of the 16 analysed PAHs (EPA 610 PAHs Mix, Supelco) (Table 2.1). Since the intersept was generally close to zero, calibration curves were forced to intercept the origin ($b = 0$).

Table 2.1 – Calibration curves for quantification of the 16 PAHs.

Compound	Calibration Curve	Correlation Coefficient
Naphthalene	y = 31613x	0.999
Acenaphthylene	y = 51706x	0.999
Acenaphthene	y = 7339x	0.988
Fluorene	y = 170669x	0.996
Phenanthrene	y = 626708x	0.998
Anthracene	y = 1423773x	0.999
Fluoranthene	y = 158442x	0.998
Pyrene	y = 131852x	0.979
Benzo[*a*]anthracene	y = 282430x	0.998
Chrysene	y = 279778x	0.999
Benzo[*b*]fluoranthene	y = 316517x	0.999
Benzo[*k*]fluoranthene	y = 340341x	0.997
Benzo[*a*]pyrene	y = 273820x	0.999
Dibenzo[*ah*]anthracene	y = 37509x	0.999
Benzo[*ghi*]perylene	y = 79470x	0.999
Indene[123-*cd*]pyrene	y = 391216x	0.999

Blanks were performed extracting an amount of sodium sulphate equivalent to that used with the samples. PAHs were not detected in the procedural blanks.

PAH measurements were validated with a reference material of sewage sludge (CRM 088) containing the PAHs most frequently detected in sediment samples. The comparison of the obtained and certified values is in Table 2.2. In general there was a good agreement between obtained and certified values, except for benzo[*a*]anthracene whose percentage of recovery was around 150%.

Table 2.2 –PAH concentrations in CRM 088 (sewage sludge). Significantly different values are marked.

	CRM 088 (µg g^{-1} dw)	
Compound	**Obtained values**	**Certified values**
Pyrene	2.04 ± 0.09	2.16 ± 0.09
Benzo[*a*]anthracene	1.44 ± 0.03*	0.93 ± 0.09
Benzo[*b*]fluoranthene	1.06 ± 0.05	1.17 ± 0.08
Benzo[*k*]fluoranthene	0.66 ± 0.06	0.57 ± 0.05
Benzo[*a*]pyrene	0.92 ± 0.14	0.91 ± 0.09
Indene[123-*cd*]pyrene	0.73 ± 0.46	0.81 ± 0.06

The detection limit (Table 2.3) was determined calculating the amount of sample needed to give a peak with 3 times the height of the chromatogram baseline noise and divided by the average amount of sediment used in the extraction (Lindsay, 1992).

Solvents (trace organic analysis grade) were purchased from Merck with the exception of hexane that was purified in the laboratory by a fractional distillation using a Dufton column. Its purity was confirmed, after concentration, by GC-FID analysis in a Hewlet Packard 6890 instrument. Acetonitrile was HPLC grade (Merck) and the water used in the HPLC procedure was deionised in a Mili-Q System.

Table 2.3 – Detection limit of individual PAHs.

Compound	Detection Limit ($ng\ g^{-1}$)
Naphthalene	0.11
Acenaphthylene	0.13
Acenaphthene	0.24
Fluorene	0.02
Phenanthrene	0.01
Anthracene	0.01
Fluoranthene	0.02
Pyrene	0.02
Benzo[*a*]anthracene	0.01
Chrysene	0.01
Benzo[*b*]fluoranthene	0.01
Benzo[*k*]fluoranthene	0.01
Benzo[*a*]pyrene	0.01
Dibenzo[*ah*]anthracene	0.06
Benzo[*ghi*]perylene	0.03
Indene[123-*cd*]pyrene	0.01

2.2.3. Organic carbon content

Three sub-samples (approximately 10g) of each sediment sample were air-dried to constant weight for the determination of the wet and dry weight ratio.

The organic carbon content was determined in the dried samples by burning at 500ºC in a furnace for 15 minutes. Organic carbon content was determined by weight loss.

2.2.4. Statistical Analysis

Data are presented as mean ± one standard deviation. Parametric one-way analysis of variance test with *post-hoc* comparisons was used to test differences between groups when homogeneity of variances (Levene's test) and normality (Shapiro Wilk test) prevailed. In other cases, nonparametric Kruskall-Wallis and Mann-Whitney U-test were applied. The differences

were considered statistically significant when $p<0.05$. Computer program STATISTICA for Windows Release 5.1 (StatSoft, Inc., Tulsa, OK) was used.

Spatial and seasonal variability in the data was also assessed with Principal Components Analysis (PCA) using the means. PCA is used to transform an original set of potentially correlated variables into a reduced set of uncorrelated variables, which are called Principal Components. These components are obtained in order of decreasing importance. The first Principal Component explains the most variance; the second Principal Component explains the next, and so on. For each sample PCA calculates a "score" and two-dimensional plots of the scores for the first two Principal Component reveal clusters and trends in the data.

Other multivariate analysis techniques may be used to identify the major contributing sources and explain data groupings. Partial Least Square (PLS) Analysis is an extension of a PCA where a projection model is developed in order to predict Y from X using the PCA loadings of X. In essence, PLS performs a PCA on data that are defined as the signature. This dataset which can be chemical, physical or biological in nature is called the X-Block and ideally will be a pure source sample but could be made up of environmental samples that have a high proportion of a single source such as fuel spills or soots. Since the samples come from the same source, although the concentrations may vary, PCA will generate a Principal Component 1 (PC 1) that explains most of the variance in the data, typically >90%. This projection or vector in n-dimensional space where n is the number of chemical compounds analysed, can be described by a series of loading factors on each compound; those compounds which have a major impact on PC 1 will have high loadings (either positive or negative) whereas those compounds which are relatively unimportant and, therefore, do not have a major influence on the data, will have values close to zero. PC 2 is fitted orthogonal to the first component so there is no component of PC 1 influencing PC 2. Once the first two PCs have been elucidated, their projection can be described in terms of the two sets of loadings. These projections, which represent the signature defined in

terms of the chemical compounds used, can now be applied to the environmental data (Y-Block). The amount of variance explained or predicted by each X-Block signature can be quantified. This can be shown graphically either through a scatter plot of the weightings on each sample or as the total variance explained. If the signature is similar to that of the environmental data, a high value for the explained variance is produced. Conversely, if a poor fit is produced, the explained variance is also small. Each signature can be fitted in turn and all are fitted independently of each other. If none of them explain the variation seen in the data, the fits will be small in every case (Mudge, 2002).

Simca-P, version 10.0.2.0 (Umetrics) was used to perform the multivariate analysis.

2.3. RESULTS

Figure 2.5 presents the spatial and seasonal variations in tPAH concentrations (sum of the 16 individual analysed PAHs) in the surface sediments. The individual PAHs concentrations are presented in Table A2 in Annexe.

Mean tPAH concentrations in the sediments varied between 1.01±0.07 and 66.3±6.4 $ng.g^{-1}$ dw. Acenaphthene (0.44±0.05 – 13.3±0.5 ng g^{-1} d.w.) and fluoranthene (0.07±0.00 – 9.90±1.22 ng g^{-1} d.w.) were the most abundant PAHs at all sites.

Spatially, mean tPAH concentrations were significantly higher in sediments from sites 1 and 6, but only from January to May (winter and spring; Figure 2.5). In June, the highest tPAH concentrations were at site 4 and in July at site 7, while in August no significant differences were observed between sites (p>0.05).

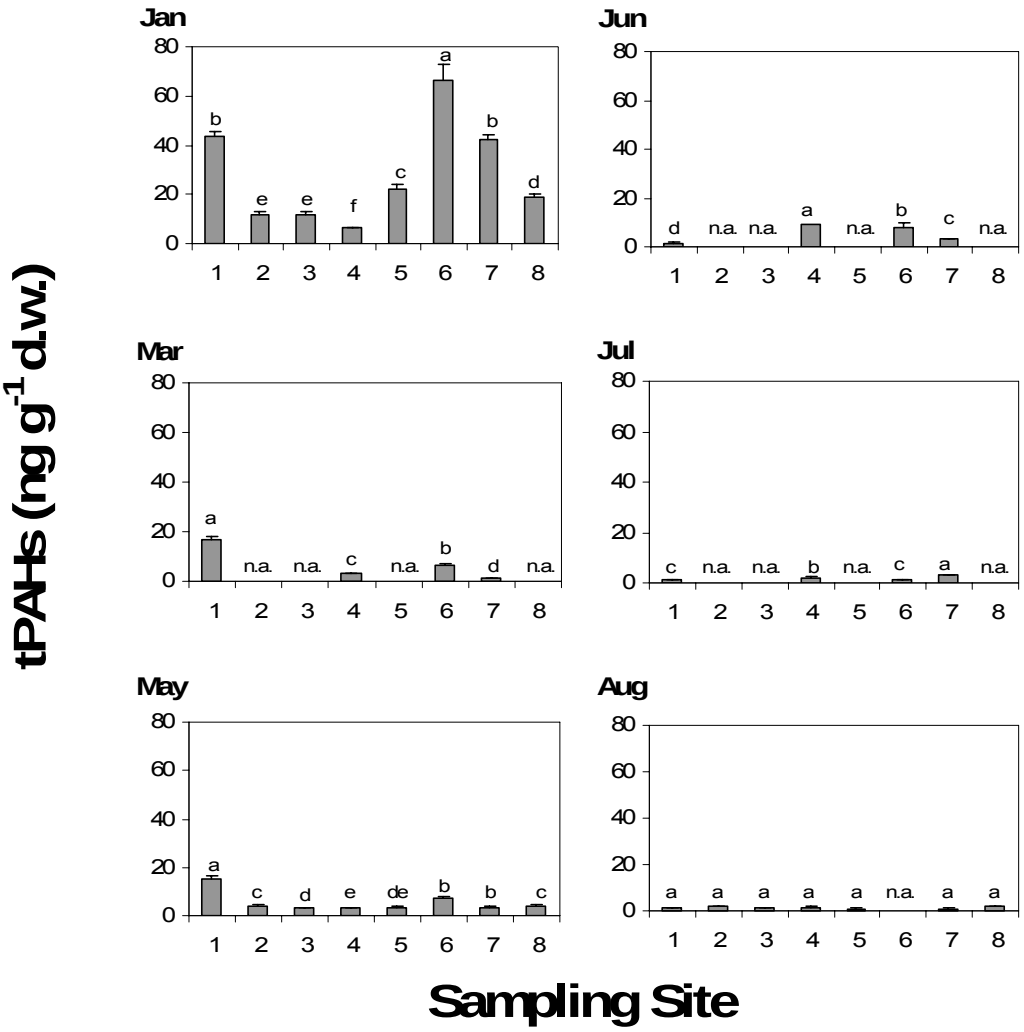


Figure 2.5 – Spatial and seasonal variation (mean ± standard deviation) of tPAH concentrations in sediments from different sites from the Ria Formosa. Bars labelled with the same letter are not statistically different ($p < 0.05$). n.a. – data not available.

Seasonally, the highest mean tPAH concentrations in the sediments were in January, ranging from 6.35±0.43 to 66.3±6.4 ng g^{-1} d.w. (Table A2). The most abundant individual PAHs, in January, were fluoranthene (0.24±0.07 – 9.90±1.22 ng g^{-1} d.w.), pyrene (1.24±0.22 – 5.87±0.08 ng g^{-1} d.w.) and PAH acenaphthene (1.65±0.39 – 13.3±0.5 ng g^{-1} d.w.).

In the following months (March – August), tPAH concentrations were significantly lower. The lowest mean tPAH values were in August (1.01±0.06 – 1.84±0.07 ng g^{-1} d.w.). The most relevant individual PAHs during this period were acenaphthene (0.44±0.05 – 9.47±2.33 ng

g^{-1} d.w.), fluorene (0.18±0.03 – 2.44±0.25 ng g^{-1} d.w.), phenanthrene (0.14±0.04 – 1.74±0.02 ng g^{-1} d.w.) and pyrene (0.08±0.01 – 1.69±0.30 ng g^{-1} d.w.) (Table A2).

Individual PAHs were also grouped by aromatic ring number (Figure 2.6). The 2+3 ring PAHs comprises naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene and anthracene. The 4 ring PAHs group encloses fluoranthene, pyrene, benzo[*a*]anthracene and chrysene. Finally, the 5+6 ring group is constituted by benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenzo[*a,h*]anthracene, benzo[*g,h,i*]perylene and indene[123-*cd*]pyrene. The distribution of individual PAHs in January was significantly different from the other months ($p<0.05$). In this month, the 4 ring PAHs were the most abundant (46 – 64%) at all sites, with few exceptions. These were at sites 3 and 4 where 2+3 ring PAHs were the most abundant hydrocarbons, and at site 6 where the 5+6 rings hydrocarbons completely dominated the PAHs distribution.

The most abundant PAHs in the 4-ring group were fluoranthene and pyrene and in the 2+3 ring group it was acenaphthene (Table A2). The highest abundance of the 5+6 rings in site 6 was mostly due to the unusual high concentration of dibenzo[*a,h*]anthracene (26.5 ng g^{-1} d.w.).

In March, however, the 2+3 ring PAHs were the most abundant at sites 1 and 6 (73 – 75%), especially due to acenaphthene, while the 4 ring group dominated at sites 4 and 7 (80 – 90%), mostly due to pyrene. The 5+6 rings PAHs were generally undetected.

In May, the distribution of individual PAHs was dominated by the 2+3 ring group at all sites (54 – 88%) with relatively low contributions from the other PAHs. Acenaphthene was, in all cases, the most abundant PAH.

In June, however, the 2+3 ring PAHs were the most abundant only at sites 4 and 6 (76 – 77%). In the remaining sites the 4 ring PAHs were predominant (53 – 72%).

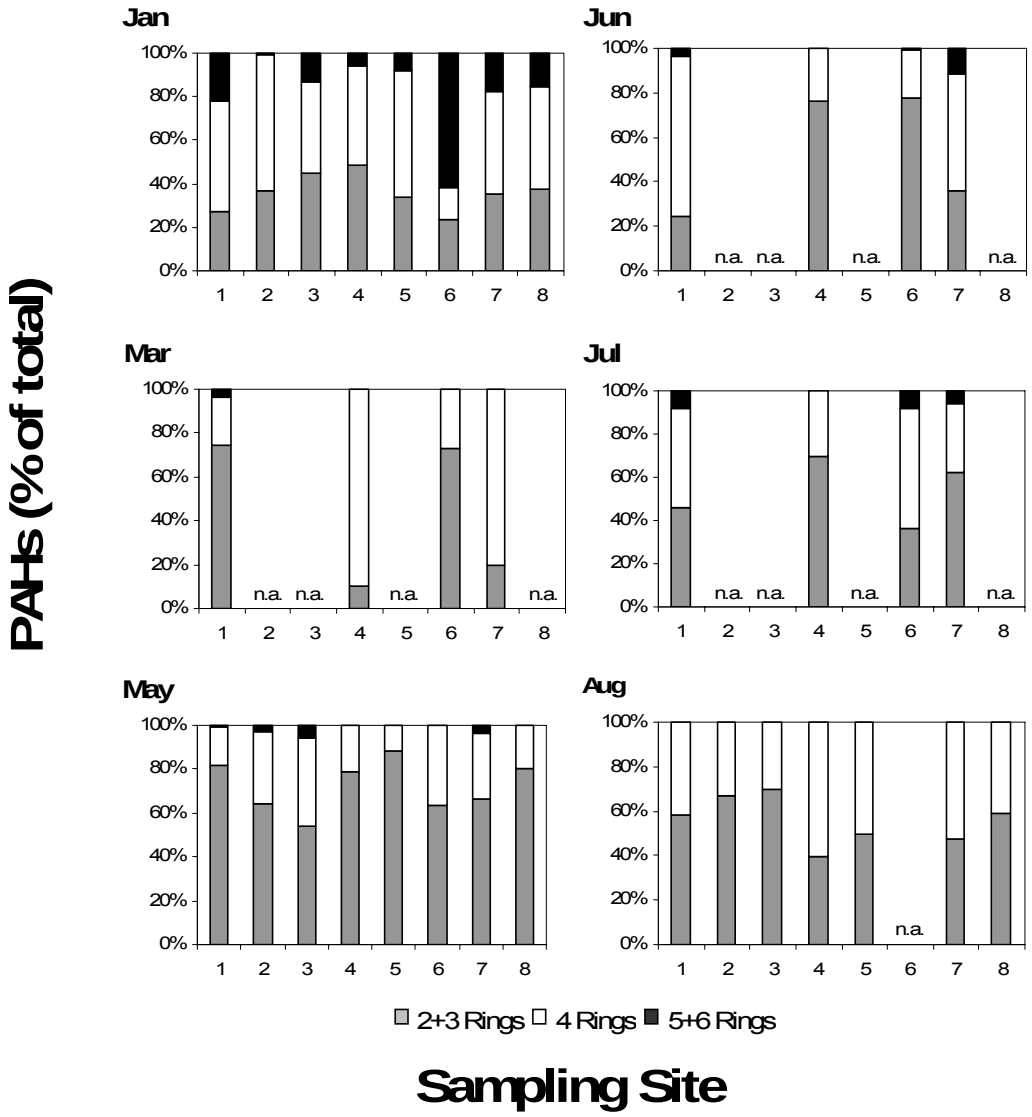


Figure 2.6 – Sediment distribution pattern of individual PAHs grouped by number of aromatic rings. n.a. – data not available.

Contrary to the previous month, in July, the 2+3 ring PAHs were dominant in sites 4 and 7 (63 – 70%) while the 4 ring PAHs were predominant at sites 1 and 6 (46 – 56%). As in May, the 5+6 ring group was not relevant.

In August, the 2+3 rings PAHs were slightly more abundant than the 4 ring PAHs (55 vs 45%, respectively); the 5+6 rings were completely absent. The most abundant PAHs were acenaphthene, fluorene and phenanthrene (see Table A2).

Table 2.4 presents the organic carbon content (OC) of the sediments. The sediment organic carbon content ranged from 0.6 to 3.4%. The highest OC was in the sediments collected

in January and was significantly different from the other months (p<0.05). Spatially, the OC was highest in the sediments from sites 1 and 7.

Considering all samples together and relating them to the PAH content, tPAH sediment concentrations were directly related to the organic carbon content (tPAH (sed) = 13.13 OC – 10.94; r = 0.71, n = 35, p < 0.01) (Figure 2.7), which indicates that the organic carbon content may be influencing the PAH distribution in the Ria Formosa.

Table 2.4 – Sediment organic carbon content (%).

Site / Month	1	2	3	4	5	6	7	8
January	3.18	2.63	1.39	2.10	1.66	2.97	3.40	2.44
March	1.99			1.58		1.76	1.87	
May	1.88	0.83	1.29	1.14	0.88	0.75	2.77	1.15
June	1.01			0.68		0.98	2.00	
July	1.23			0.80		1.04	1.68	
August	1.71	1.33	0.84	1.11	0.83		0.99	0.81

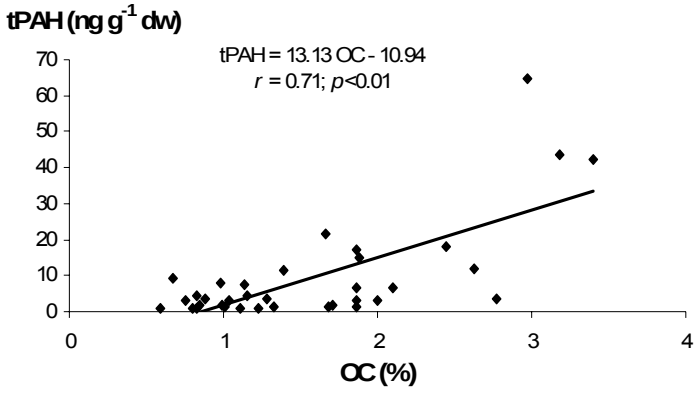


Figure 2.7 – Relationship between sediment tPAH concentration (ng.g^{-1} dw) and their organic content, OC (%).

A Principal Component Analysis performed on the individual PAH concentrations in the sediments, using the sediment data expressed as a proportion of the tPAH is shown in Figure 2.8. The centred log ratio procedure (normalisation first to the concentration total and then to the

geometric mean) was used to produce a normalised data set that was not affected by negative bias or closure (Yunker & Macdonald, 2003). Data were autoscaled and log transformed before PCA. In this analysis, PC 1 explained 41.7% and PC 2 explained 26.9% of the variance in the data.

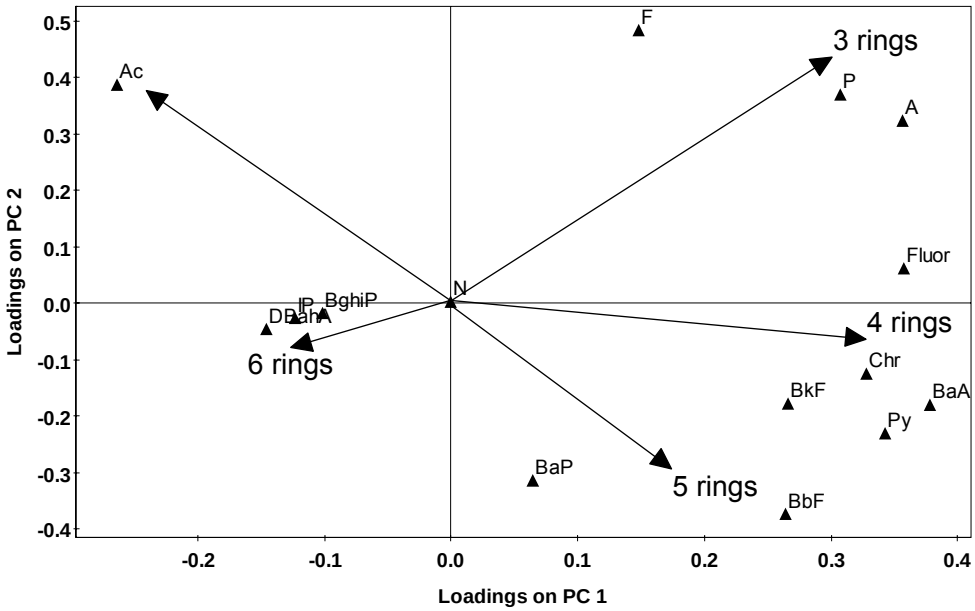


Figure 2.8 – PCA of the individual PAH (as a proportion of tPAH) in the sediments, showing the loadings on PC1 and PC2. PC1 represents 41.7% of the variance and PC2 26.9% of the total variance of the data, both being significant (p<0.05; n = 36). Data were log transformed and autoscaled.

In this plot, compounds are arranged by number of rings and five vectors may be drawn indicating the positioning of the several groups. One vector points to acenaphthene, which loads negatively in PC 1 and positively in PC 2. The second vector points to a group of 3 aromatic rings composed by fluorene, phenanthrene and anthracene, all located in the positive part of PC 1 and PC 2. The third and fourth vectors indicate the 4 and 5 aromatic ring PAHs respectively. They both load positively in PC 1 and negatively in PC 2. Finally, the fifth vector points to the 6 aromatic ring PAHs group, which load negatively on both PC 1 and PC 2.

The combination of these variables resulted in a scores plot where similarity and grouping of samples are presented. Figure 2.9 shows the scores plot, with the samples labelled by sampling month with the loading vectors overlaid. Most of the January samples load in the positive direction of PC 1 and negative of PC 2 towards the bottom right of the figure, implying that they are highly influenced by the high molecular weight hydrocarbons (4 and/or 5 aromatic rings); the May samples are located in the upper left, meaning that they are dominated by acenaphthene, with a small contribution from the 6 aromatic ring PAHs; the August samples load positively on both PC 1 and PC 2, which presents a mixed composition of the 3 aromatic rings fluorene, phenanthrene and anthracene. March, June and July samples are scattered and do not group, however, their position in the scores plot suggests a mixed PAHs signature.

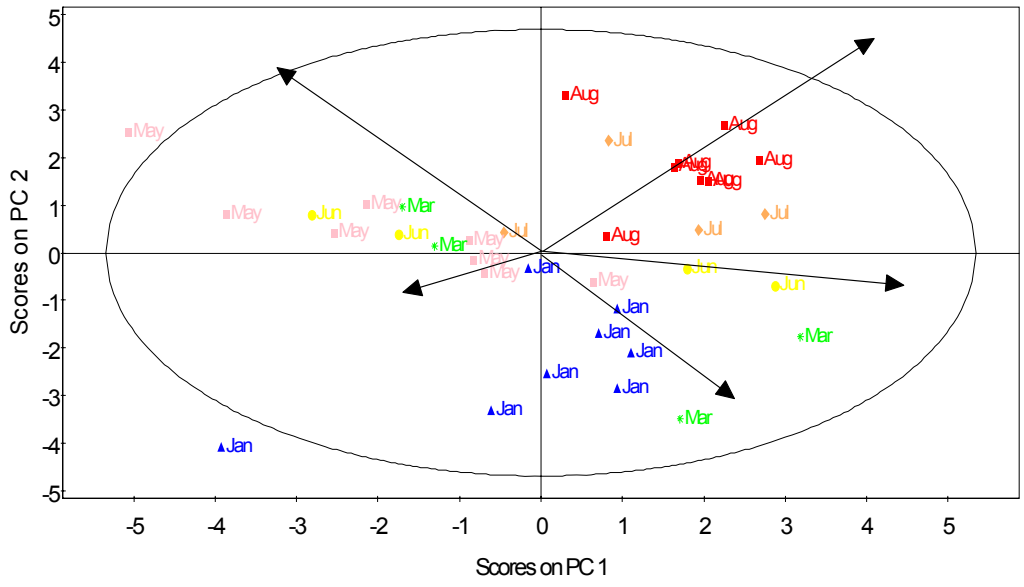


Figure 2.9 – PCA of the individual PAHs as a proportion of the tPAH, in the sediments, showing the data scores labelled as month of sampling.

In this diagram, the circle line represents the Hotelling's T^2, defining the circular 95% tolerance (Eriksson *et al.*, 1999). In this diagram, there were two samples (Jan 6 and May 5), which fall outside this limit. That is, their behaviour is very dissimilar to the other samples. In

this case, Jan 6 sample presents a signature enriched in the 6 aromatic rings PAHs while in May 5 the sediments are more enriched in acenaphthene.

The same scores may be visualized by sample location (Figure 2.10). In this figure, there is no clustering of samples according to the sampling site.

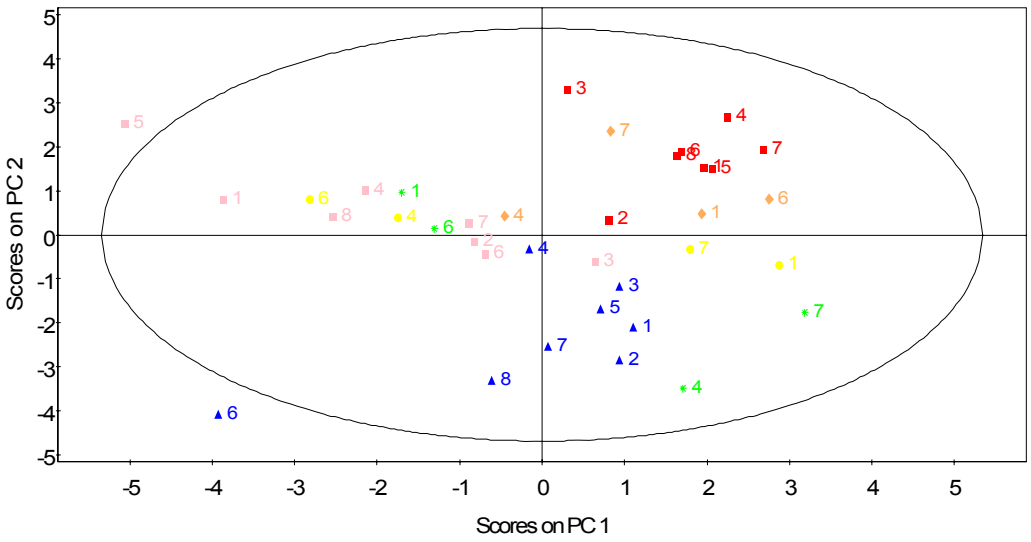


Figure 2.10 – PCA of the individual PAH as a proportion of the tPAH, in the sediments, showing the data scores labelled as sampling site number. Colours indicate sampling month.

The molecular ratios phenanthrene/anthracene (P/A), fluoranthene/pyrene (Fluor/Py) and low molecular weight (2+3 ring PAHs)/high molecular weight (4+5+6 ring PAHs) (2+3/4+) were also calculated for the sediment PAH data (Table 2.5). The P/A ratio is generally lower or close to 10, indicating that none of the samples has a marked petrogenic origin (P/A ratios higher than 10). There is also no evidence of spatial or seasonal variation. The Fluor/Py ratio varies between 0.11 and 2.79. Most of the January, May, July and August samples present ratio values greater than 1, which generally points to a pyrolytic origin. In the same way, sites 1, 3, 5 and 8 present always a Fluor/Py ratio higher than 1. The low/high molecular weight ratio shows also a seasonal variation, with the ratios being significantly lower in January and higher in May ($p <$

0.05). This observation is not consistent with the Fluor/Py ratio, since the high values of the low/high molecular weight ratio found in May are usually associated with petrogenic origin.

Table 2.5 – Phenanthrene/Anthracene (P/A), Fluoranthene/Pyrene (Fluor/Py) and Low/High molecular weight ratios for the sediment samples.

	Site							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
P/A Ratio								
January	3.98	8.19	4.03	2.75	2.50	11.87	3.85	3.69
March	7.79			5.54		5.45	4.05	
May	10.28	3.45	7.30	4.47	4.34	8.10	7.25	4.42
June	6.47			9.92		9.85	10.73	
July	3.44			5.70		6.43	6.16	
August	4.97	8.36	9.79	4.53	4.36	-	6.74	6.92
Fluor/Py Ratio								
January	1.69	0.51	1.35	0.11	1.08	0.40	1.55	1.35
March	1.98			0.17		0.64	0.54	
May	1.30	1.35	1.02	2.35	1.84	1.32	0.98	1.23
June	0.94			0.85		0.88	0.96	
July	1.36			1.04		1.80	1.85	
August	1.20	0.54	2.07	2.79	1.31	-	1.82	1.46
Low/High Ratio								
January	0.16	0.21	0.29	0.33	0.21	0.12	0.22	0.22
March	2.96			0.12		2.68	0.25	
May	4.59	1.79	1.17	3.78	7.69	1.75	1.96	3.99
June	0.32			3.17		3.44	0.56	
July	0.86			2.28		0.55	1.67	
August	1.41	2.05	2.29	0.65	0.98	-	0.90	1.43

Low = 2+3 aromatic rings PAH and High = 4+ aromatic rings PAH.

The schematic representation of the P/A versus the Fluo/Py ratios usually helps to identify the PAHs origin (Figure 2.11). In this plot, the majority of the samples, present P/A ratios < 10 and Fluo/Py ratios > 1 characteristic of a pyrolitic origin.

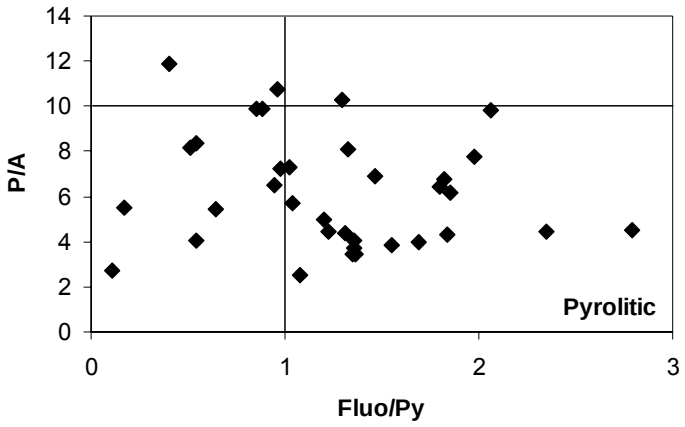


Figure 2.11 – P/A versus Fluo/Py ratio for the sediment samples.

In order to clarify the origin of the PAH contamination in the Ria Formosa sediments, Partial Least Squares Analysis (PLS) was also used. The lack of studies concerning the individual PAH concentrations in the Ria Formosa was a major difficulty in this analysis. Therefore, nineteen PAH signatures from other locations (*e.g.* Mediterranean, Alaska) were taken from the literature and used to develop an X-block in PLS. Although these signatures may not have a direct relevance here, they are indicative of different types of PAH sources. Table 2.6 presents the individual PAH amounts in the several signatures used.

Each signature was fitted in turn to the sediment PAHs data and the amount of variance explained by the most relevant signatures is presented in Figure 2.12 as stacked bars (see section 2.2.4. for detailed explanation).

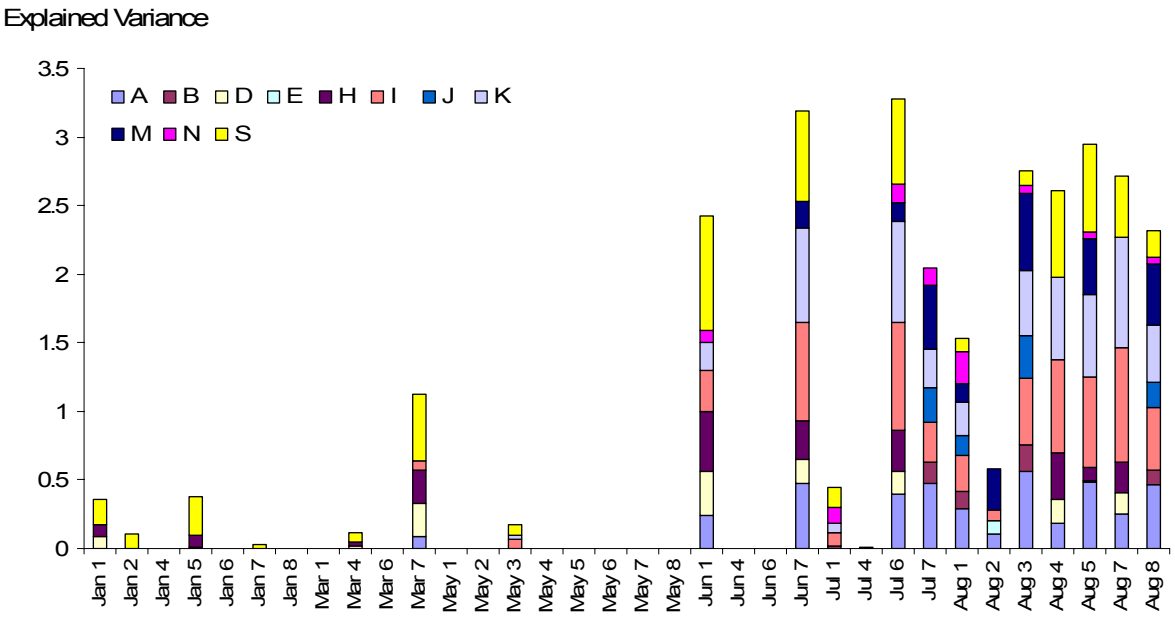


Figure 2.12 – Partial Least Squares analyses of the PAH signatures in the sediment data (15 *observations*; 91 *variables*). Nineteen different PAHs source signatures, taken from the literature were used to develop the X-block.

Most of the variance in the sediments data is not explained by the signatures used, or the amount of variance explained is very low, meaning that the PAHs proportion in the sediments is not similar to any of the signatures used. These occur mainly in January, March and May. Most samples, however have explained variances that exceed 1. This implies that there is a signature overlap between the sources used, which is not unexpected since several of the signatures may have common features.

The signatures that better explain the variance in the sediments data were signatures A (background pre-spill – petrogenic signature), H (sediment affected by combustion products), I (creosote – pyrolytic signature), K (diesel soot – pyrolitic signature), M (very lightly weathered Exxon Valdez oil) and S (tar – petrogenic signature). They occur in almost all the sediment samples collected in the summer months of June, July and August.

Table 2.6 – Proportion of PAH in samples or averages of samples that define the major PAH sources analysed.

	A*	B*	C*	D*	E*	F*	G*	H*	I**	J**	K**	L**	M*	N*	O*	P*	Q*	R*	S*
N	11	67	7.8	-	160	4.1	410	45	3213	745	-	622	43	-	-	0.9	3.6	-	1.0
Ac	2.5	4.0	-	-	13	0.4	140	62	4695	-	-	2.0	-	-	-	-	-	-	3.0
F	18	29	1.8	-	85	1.1	170	129	8137	449	1.5	93	120	950	-	1.2	-	-	4.0
P	55	111	7.8	1869	1200	7.5	3000	1039	77033	1330	16	262	340	3900	66	6.1	9.6	9.9	27
A	2.0	8.0	1.2	446	240	2.1	580	162	1176	59	1.0	-	-	-	31	-	-	-	3.0
Fluor	10	8.0	-	3183	3700	20	5000	1467	44535	-	7.6	2.0	-	-	26	7.3	-	-	26
Py	15	12	2.0	2804	4600	19	4000	1167	27188	27	6.0	10	13	390	35	-	-	-	27
BaA	6.0	9.0	-	1596	4300	15	1300	469	3206	-	-	2.0	9.3	-	-	-	-	-	6.0
Ch	19	22	-	1756	5100	16	2300	755	11915	2.0	2.7	46	110	3000	530	190	55	36	13
BbF	15	6.0	1.2	2769	10000	15	6000	1135	4047	-	2.4	6.0	3.5	390	-	29	13	9.4	6.0
BkF	0.5	1.0	-	-	2300	14	960	-	2936	-	0.6	-	16	-	-	-	-	-	1.0
BaP	3.0	4.0	-	1607	6800	20	1900	492	1170	-	-	-	22	770	140	2.5	7.5	4.4	2.0
DBA	2.5	1.0	-	247	680	4.1	460	196	270	-	-	1.0	-	-	-	5.3	-	-	2.0
BPer	13	4.0	-	1028	4500	12	3900	503	1121	-	-	2.0	5.3	150	37	21	25	8.8	4.0
IP	4.0	1.0	-	1296	5200	11	3000	436	1341	-	1.0	1.0	-	-	-	-	-	-	1.0

* ng g^{-1}; ** mg kg^{-1}.

Signature A: background PAHs found in a combination of several pre-spill layers of subtidal box core samples (Page et al. 1999).

Signature B: background PAHs in sediments off-shore Yakataga (Burns et al. 1997).

Signature C: background PAHs in sediments off-shore Port Chalmers (Burns et al. 1997).

Signature D: PAHs in a sediment sample from the Ajaccio Harbour severely exposed to PAHs from boat traffic (Baumard et al. 1998a).

Signature E: PAHs emitted in forest fires with a mixed oak and pine origin (Burns et al. 1997).

Signature F: PAHs in sediments affected by combustion products from human habitations (e.g., ash, soot and smoke from wood-burning fireplaces, charcoal burning) (Burns et al. 1997).

Signature G: atmospheric dust standard reference material NIST-SRM 1649 (Page et al. 1999).

Signature H: average of 29 NOAA sediment trap samples produced in the open burning of coniferous softwood (Page et al. 1999).

Signature I: PAHs from a piling at New Chenega Village in Sawmill Bay (Page et al. 1999).

Signature J: Alaska diesel oil (Burns et al. 1997).

Signature K: diesel soot formed during the combustion of Alaska diesel oil by the sampling ship Glorita (Burns et al. 1997).

Signature L: fresh Alaskan North Slope oil (Burns et al. 1997)

Signatures M to R: PAHs found in sediments contaminated by the Exxon Valdez spill (Alaska North Slope oil) subjected to different weathering processes, from very lightly weathered (M) to extremely weathered (R) (Burns et al. 1997; Page et al. 1999).

signature S: average of three samples of weathered surface tar collected at Peak Island (Monterey Tar) (Page et al. 1999).

2.4. DISCUSSION

There is no previous information about total or individual PAHs concentrations in the sediments of the Ria Formosa lagoon. Nevertheless, the values presented here are similar to those typical of coastal areas and are considerably lower than the ecotoxicological assessment criteria (see Table A3 in Annexe) defined for sediments by the OSPAR Commission (OSPAR Commission, 2000). According to the classification used by Notar *et al.* (2001), the sediments from the Ria Formosa lagoon may be considered slightly contaminated (tPAH < 250 ng g^{-1}) when compared with PAH concentrations in sediments from different European locations (see Table 1.3 in Chapter 1). In fact, tPAH concentrations in the sediments of the Ria Formosa were of the same order of magnitude as those collected in the central part of the Gulf of Trieste (Notar *et al.*, 2001), or in remote locations or slightly urbanised and leisure areas of the Mediterranean Sea (Baumard *et al.*, 1998b).

The highest sediment PAH concentrations were in January and a decreasing trend was observed until a minimum was reached in the summer (July and August). This seasonal variation was linked to a seasonal variation in PAH sources. In fact, Principal Components Analysis indicated that the variation in source changed with season but was common to all the sampling sites (Figure 2.9 and 2.10).

The greatest PAH concentrations in the winter, could be associated with an increase in anthropogenic PAHs including domestic heating (Launhardt & Thoma, 2000; Zou *et al.*, 2003), urban run-off (McCready *et al.*, 2000; Marttinen *et al.*, 2003) and river discharges (Mitra & Bianchi, 2003), which are frequently highest in winter (Mangani *et al.*, 2005; Prevedouros *et al.*, 2003). In fact, in the period of sampling, rainfall was highest in December-January (140 to 160 mm) (http://snirh.inag.pt). These anthropogenic PAH emissions could explain the greater PAH

concentrations at sites 6 and 7 in winter, as they are located near urban centres (Olhão and Tavira, respectively). Site 7 is influenced by the River Gilão which is the only constant freshwater input to the lagoon. This river receives the discharge from a domestic sewage treatment plant from the city of Tavira (serving a population of ≈ 20 000 habitants) thus being also a source of PAHs to the lagoon (Instituto da Água, 1994; Marttinen *et al.*, 2003). Increased rainfall and river flow are associated with increased amounts of suspended matter entering the lagoon. Small increases in the suspended matter in the Ria Formosa were reported in the winter (50 mg l^{-1}), in the vicinity of the River Gilão (Ferreira & Vale, 1995), which could be linked to the increase in PAH concentrations in the sediments, in this season, at site 7. In fact, increases in PAH concentrations in settling material were also detected near the River Gilão outlet, in the winter, which were even higher than those found in the sediments (Veriato *et al.*, 1998). Sites 6 and 7 are also influenced by Olhão and Tavira fishing and recreational ports, which have a capacity for around 2000 and 1000 fishing and recreational boats respectively. Boats are considered an important source of pollutants, including PAHs, to the marine environment (Kado *et al.*, 2000; Kelly *et al.*, 2005). Most boats and ships (including other watercrafts as Jet Skis) emit part of their fuel/oil mixture and exhaust gases into the water. Engine emissions comprise a mixture of PAHs derived from several compartments including those initially present in the fuel, formed during the combustion process, or accumulated in the lubricating oil and present in the exhaust system (Marr *et al.*, 1999; Schaeur *et al.*, 2002; Lima *et al.*, 2005). Such emissions may be strongly affected by the type of engine (spark ignition, diesel), age of the motor, vehicle speed, type of fuel, etc. (Schaeur *et al.*, 2002; Lima *et al.*, 2005). For example, an outboard two-stroke engine operated at 60% throttle will emit ≈ 900 µg PAHs kW^{-1} h^{-1} into seawater (Kelly *et al.*, 2005). In this way, it could be roughly estimated that 2000 boats (the capacity of Olhão harbour) with an average 75 horse-power engine (55.2 kW) operating at 60% throttle will emit ≈100 g PAHs h^{-1} into the environment. However, without the knowledge of each boat specific

characteristics, speed and amount of time spent in the Ria Formosa it is very difficult to quantify the exact amount of PAHs release by boat traffic into this aquatic environment.

The elevated tPAH concentrations at site 1 in January (Figure 2.5) are not explained by the sources of sites 6 and 7, since it is not located close to any relevant population centre. However, site 1 is located near the main entrance of the Ria Formosa, used by all commercial ships going in and out of the commercial harbours of Faro and Olhão, which received around 182 commercial vessels with a total tonnage of 449 879 tons in 1994, and 165 vessels with a total tonnage of 403 466 tons in 1995. Therefore, shipping is not trivial in this region and may be an important source of PAHs to the marine environment (Baumard *et al.*, 1999a).

The PAHs source allocation in the Ria Formosa was investigated using different strategies: analysis of the individual PAHs distribution pattern (Figure 2.6), analysis of specific individual PAHs ratios (P/A, Fluor/Py and low/high molecular weight PAHs; Table 2.5) and PLS analysis (Figure 2.12). However, their results were not always in agreement with each other.

The signature of the January samples suggests pyrolytic PAH sources, petrogenic in May and August and mixed in the other months (Figure 2.6). This was further supported by the low/high molecular weight PAH ratio (Table 2.5) that was lowest in January and highest in May. The other ratios, P/A ratio < 10 and Fluor/Py ratio > 1, are, however, characteristic of pyrogenic PAH sources for most of the samples (Table 2.5). These conditions were observed in almost all samples suggesting that PAHs in the sediments were of organic matter combustion origin, although the predominance of the 2+3 aromatic rings observed in May and August indicates a petrogenic PAHs source (Notar *et al.*, 2001). PAHs ratios, however, may be modified by bacterial degradation. For instances, while most PAHs are biodegraded to some extent in aerobic conditions, recent studies have demonstrated that under anaerobic conditions there is a selective degradation of phenanthrene comparing with other PAHs (Lei *et al.*, 2005). Under these

conditions, P/A ratios would be lower than expected. This highlights the need of different analysis when attempting to find the source of a specific PAHs signature.

The PAH sources assessed by the PLS approach did not, however, clarify the origin of the PAHs signatures in all samples, particularly in January when the available signatures were unable to explain most of the variance in the sediment data. The only relevant signature in January was "weathered tar" (S) explaining around 10 to 30% of the variance in the PAH data at sites 1, 2 and 5. This type of signature may be associated to road run-off of asphalt type materials, removed from the catchments. These materials may than enter the lagoon during periods of heavy rain. The sources previously mentioned, in January, were not confirmed, however.

In the summer, the A, H, I, K, M and S signatures appear as the major PAHs sources in these samples. This indicates that the individual PAHs distribution pattern is consistent with the mixed petrogenic and pyrogenic sources of the PAHs in the sediments. Signature A is typically found in remote places containing background PAH concentrations (Burns *et al.*, 1997). In terms of individual PAHs distribution pattern it is similar to signature M (there is ≈ 80% overlap between both signatures), which indicates the presence of very lightly weathered oil (Page *et al.*, 1999). Although no severe oil spills have occurred in this area, there have been some releases of hydrocarbons by marine transportations in the South Portuguese Coast and in the Ria Formosa (CCDR, 2005). In the period of this study, at least eleven of these accidental or deliberate spills occurred in this area. The South of Portugal is also near the route of large ships going in and out of the Atlantic, being thus also subjected to several types of oil releases (Vieites *et al.*, 2004). This type of signature was found in almost all collected samples highlighting the importance of oil releases as a source of PAHs in the Ria Formosa.

Signatures I (creosote) and K (diesel soot) are closely related to boat traffic (Burns *et al.*, 1997; Page *et al.*, 1999). Although, it is impossible to determine the exact number of boats inside

the Ria Formosa, there is a large increase (around 10x) in boat traffic in the summer due to tourism and fishing activities. Another possible source for signature K (diesel soot) is the proximity of the Faro International Airport, which also shows a three fold increase in aeroplane traffic in the summer (ANA, 1994; ANA, 1995). Airports are responsible for PAH emissions by several ways, including aircraft engines, ground vehicles as well as all kinds of diesel engine powered systems and furnaces (Topal *et al.*, 2004; Zielinska *et al.*, 2004). Signature S (weathered tar) is not easily explained since it is generally associated with periods of intense rain and in the summer months, rain is not frequent. However, the weathered tar signature presents some similarity with oil signatures and maybe related to the previously mentioned hydrocarbon releases in the Ria Formosa and South Portuguese Coast (Page *et al.*, 1999; CCDR, 2005).

In conclusion, the PAHs found in the Ria Formosa sediments are seasonally dependent rather than spatially and their variation is strongly related to a change in source, which is more relevant in winter. The major sources were related to run-off in periods of intense rain (winter) and intense boat traffic (summer).

3. POLYCYCLIC AROMATIC HYDROCARBONS IN THE CLAM *Ruditapes decussatus*

3.1. INTRODUCTION

As stated in the previous chapters, polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds with two or more fused aromatic rings. PAHs may be formed by the incomplete combustion or pyrolisis of organic materials (pyrogenic PAHs), rearrangement and transformation of biogenic organic materials to form fossil fuels (petrogenic PAHs), transformation of certain classes of organic compounds in soils and sediments (diagenic PAHs) or direct synthesis by organisms (biogenic PAHs). However, combustion of organic matter is thought to be the major source of PAHs to the environment (Laflamme & Hites, 1978; Wakeham *et al.*, 1980; Gschwend & Hites, 1981; Simcik *et al.*, 1999; Slater *et al.*, 2002) (see Chapter 1.1 for detailed description on PAH sources).

PAHs reach the water surface mainly via atmospheric fallout, municipal and industrial effluents, urban run-off and oil spillage or leakage (Harrison *et al.*, 1975; Maltby *et al.*, 1995; Manoli & Samara, 1999; Ollivon *et al.*, 1999; Dickhut *et al.*, 2000; Polkowska *et al.*, 2000; Porte *et al.*, 2000; Golomb *et al.*, 2001; Menzie *et al.*, 2002). Due to their lipophilic behaviour, they adsorb onto particles and end up in the sediments (Borglin *et al.*, 1996; Dabestani & Ivanov, 1999; Kirso *et al.*, 2001). However, sediments may not accurately reflect the environmental load of PAHs as transient contamination in the water column is not often recorded in the sediments (Baumard *et al.*, 1998b). In these cases, organisms are better indicators since they accumulate and, therefore, "record" the contaminants present in the suspended particulate matter.

Bivalves are widely used as sentinels to assess pollution of the marine environment (Goldberg & Bertine, 2000; Solé *et al.*, 2000). Sessile filter-feeding or suspension-feeder bivalves and low metabolism organisms may take up and concentrate contaminants to levels

well above those present in the aquatic environment and give an idea of the bioavailable fraction of environmental PAHs (Baumard *et al.*, 1999b; Solé *et al.*, 2000).

Accumulation of PAHs by aquatic invertebrates may proceed via several pathways thus feeding behaviour and activity of benthic invertebrates may have a determinant effect on the bioaccumulation of organic compounds (Okay *et al.*, 2000). Bivalves also display seasonality in the uptake of contaminants, due to changes associated with their reproductive cycle and lipid content (Hellou *et al.*, 2002).

The clam *Ruditapes decussatus* is a suspension-feeder bivalve. Due to its ability to accumulate pollutants, this clam and other related clam species (*Tapes semidecussata* and *R. phillipinarum*) have been used as bioindicators for several environmental contaminants, including PAHs (see section 1.2.1). In the Ria Formosa lagoon this species is extensively cultured and represents 90% of the total Portuguese seafood production. Although clam production has decreased significantly in the last few years, this economic activity still involves directly or indirectly over 10,000 people in this region (see section 1.5). Although, several other inorganic (metals) and organic (PCBs, DDT and TBT) contaminants have been studied in this species and other bivalves from this area (see section 1.5), no information is available on PAH concentrations in *R. decussatus* or other shellfish species.

PAHs are human carcinogens after inhalation, dermal and oral exposure (including food uptake) (Schneider *et al.*, 2002; Binelli & Provini, 2003). For this reason, PAH concentrations were studied in several types of food, including shellfish (Pruell *et al.*, 1984; Gilroy, 2000; Kazerouni *et al.*, 2001; Anderson *et al.*, 2002; Camargo & Toledo, 2003). Because *R. decussatus* is used for human consumption it is important to assess if PAH concentrations in this clam species constitutes a human health problem

The aim of this study was to assess the bioaccumulation of PAHs by the clam *R. decussatus* from the Ria Formosa lagoon. Clams were collected in several clam farms over a

one-year period, and their tissues analysed for PAH concentrations. Spatial and seasonal variation, sources and possible risks for human consumption were assessed. PAH concentrations in the clam tissues were then related to PAH concentrations in the sediments (described in Chapter 2).

3.2. MATERIALS AND METHODS

3.2.1. Sampling

Around 30 clams of similar size (2.5 – 3.5 cm) were sampled monthly, at low tide, during one year, from August I (August 94) to August II (August 95), in the sites mentioned in Section 2.2.1 (Figure 2.1). Samples were collected at the eight sites in August I, October, November, December, January, May and August II and only at four sites (1, 4, 6 and 7) in March, June and July.

The organisms were wrapped in aluminium foil and maintained at 4ºC prior to their arrival at the laboratory where they were kept in aerated seawater for 2 days to clear gut content (Livingstone *et al.*, 1989). Samples were afterwards frozen and kept at –20ºC until analysis. In total, 65 samples were gathered, which were analysed in triplicate.

3.2.2. PAHs tissue analysis

Pools of three individuals were dissected and whole soft tissues dried and homogenized with anidrous sodium sulphate. The homogenate was Soxhlet extracted with hexane/dichloromethane (4:1), for 24 hours. The extract volume was reduced in a rotary evaporator and saponified with methanolic 6 N KOH at 30ºC for 18 h. The total organic extract was further purified by liquid chromatography using the procedure described above on

an activated silica/alumina column and determined by HPLC-UV using the same procedure as for the sediment samples (see Section 2.2.2). Figure 3.1 shows a chromatogram of a representative clam sample (from Site 5). As for the sediments (see Section 2.2.2), peak identification was assessed by comparison of retention times based on phenanthrene and confirmed by spectra comparison between 200 and 450 nm with the pure compounds. Concentration values are reported as ng g^{-1} wet weight and were determined using the peak areas at 254 nm by means of calibration curves (see Section 2.2.2 – Table 2.1) constructed for each of the 16 analysed PAHs (EPA 610 PAHs Mix, Supelco). The detection limits ranged between 0.01 and 0.24 ng g^{-1} wet weight, for individual PAHs (see Section 2.2.2). Blanks were prepared by extracting an amount of sodium sulphate equivalent to that used in the samples. PAHs were not detected in the blanks.

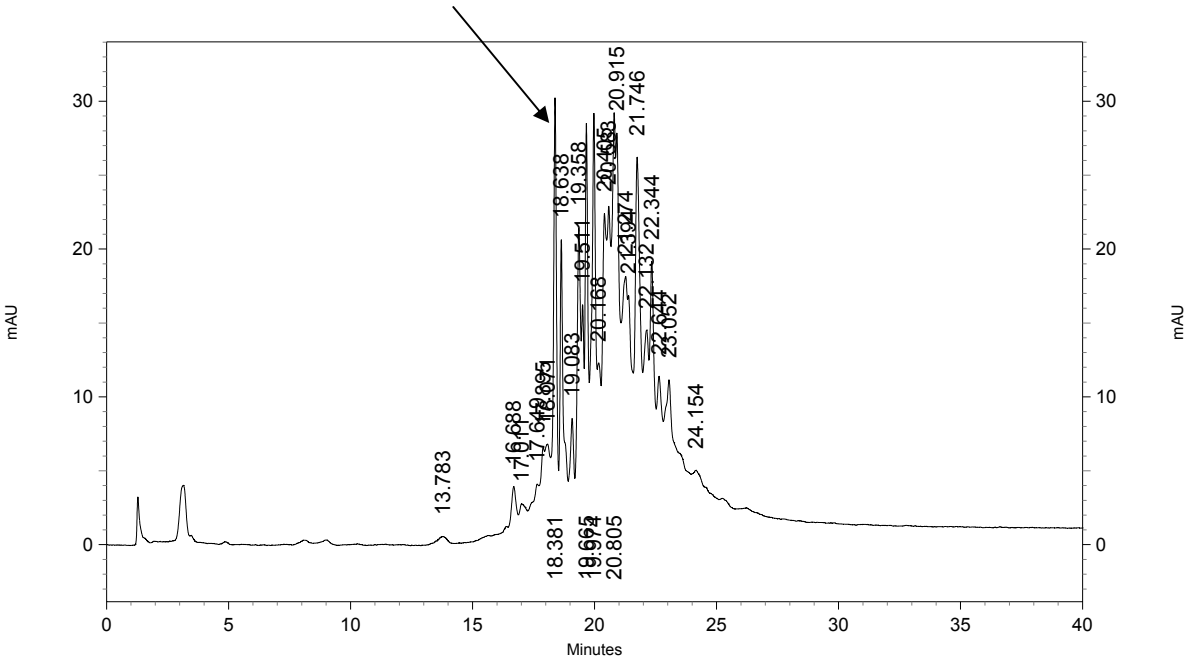


Figure 3.1 – Chromatogram of a representative clam sample (from Site 5). Phenanthrene peak is marked.

PAH measurements were validated using a reference standard material of mussel tissue (NIST 2977). The comparison of the obtained and certified values is in Table 3.1. The majority of obtained results are similar to the certified or reference (not certified) values with

the exception of fluoranthene, pyrene, chrysene and benzo[*b*]fluoranthene, whose obtained results were lower than expected.

Table 3.1 – PAH concentrations in NIST 2977 (mussel tissue). Significant differences values are marked with *.

	NIST 2977 (ng g^{-1} d.w.)	
Compound	**Obtained values**	**Certified values**
Naphthalene	23.3 ± 0.72	19 ± 5[a]
Acenaphthene	4.45 ± 0.28	4.2 ± 0.4[a]
Fluorene	10.9 ± 3.11	10.24 ± 0.43
Phenanthrene	31.7 ± 4.42	35.1 ± 3.8
Anthracene	5.80 ± 0.28	8 ± 4[a]
Fluoranthene	24.6 ± 2.62*	38.7 ± 1.0
Pyrene	43.5 ± 19.6*	78.9 ± 3.5
Benzo[*a*]anthracene	22.8 ± 3.30	20.34 ± 0.78
Chrysene	39.3 ± 6.34*	49 ± 2[a]
Benzo[*b*]fluoranthene	9.10 ± 1.32*	11.01 ± 0.28
Benzo[*k*]fluoranthene	3.98 ± 1.21	4 ± 1[a]
Benzo[*a*]pyrene	7.94 ± 1.70	8.35 ± 0.72
Dibenzo[*ah*]anthracene	1.28 ± 0.20	1.41 ± 0.19
Benzo[*ghi*]perylene	8.73 ± 1.01	9.53 ± 0.43
Indeno[123-*cd*]pyrene	4.20 ± 1.10	4.84 ± 0.81

* Significantly different

[a] Reference values (not certified)

BaP equivalents were calculated for each individual PAH by multiplying their concentration by equivalent factors determined by their toxicity (Table 3.2). Only the 4+ aromatic ring PAHs are included, since these are considered the most toxic (Gilroy, 2000).

Table 3.2 – Benzo[*a*]pyrene (BaP) equivalents for polycyclic aromatic hydrocarbons (PAH). Adapted from Gilroy (2000).

PAH	BaP equivalent
Fluoranthene	0.02
Pyrene	0.13
Benzo[a]anthracene	0.014
Chrysene	0.013
Benzo[*b*]fluoranthene	0.11
Benzo[*k*]fluoranthene	0.07
Benzo[*a*]pyrene	1
Dibenz[*ah*]anthracene	1.05
Benzo[*ghi*]perylene	0.03
Indeno[123-*cd*]pyrene	0.25

3.2.3. Tissue lipid content

The tissue lipid content was determined by the weight of the hexane/dichloromethane extracted components. Values are reported, in wet weight basis, as a percentage of the total weight tissue (Solé *et al.*, 1994).

3.2.4. Statistical Analysis

Data are presented as mean ± standard deviation. Parametric one-way analysis of variance test with post-hoc comparisons was used to test differences between groups when homogeneity of variances (Levene's test) and normality (Shapiro Wilk test) prevailed. In other cases, nonparametric Kruskall-Wallis and Mann-Whitney U-test were applied. The differences were considered statistically significant when $p<0.05$. Computer program STATISTICA for Windows Release 5.1 (StatSoft, Inc., Tulsa, OK) was used.

Spatial and seasonal variability in the data were also assessed by Principal Components Analysis (PCA) using mean values. PLS analysis was used to identify the major contributing sources and explain data groupings (see Section 2.2.3 for detailed explanation).

3.3. RESULTS

3.3.1. PAHs concentrations in the clams whole soft tissues

The spatial and seasonal variation of the tPAH concentrations in the clam whole soft tissues are in Figure 3.2. The individual PAHs concentrations are in Table A4 in Annexe.

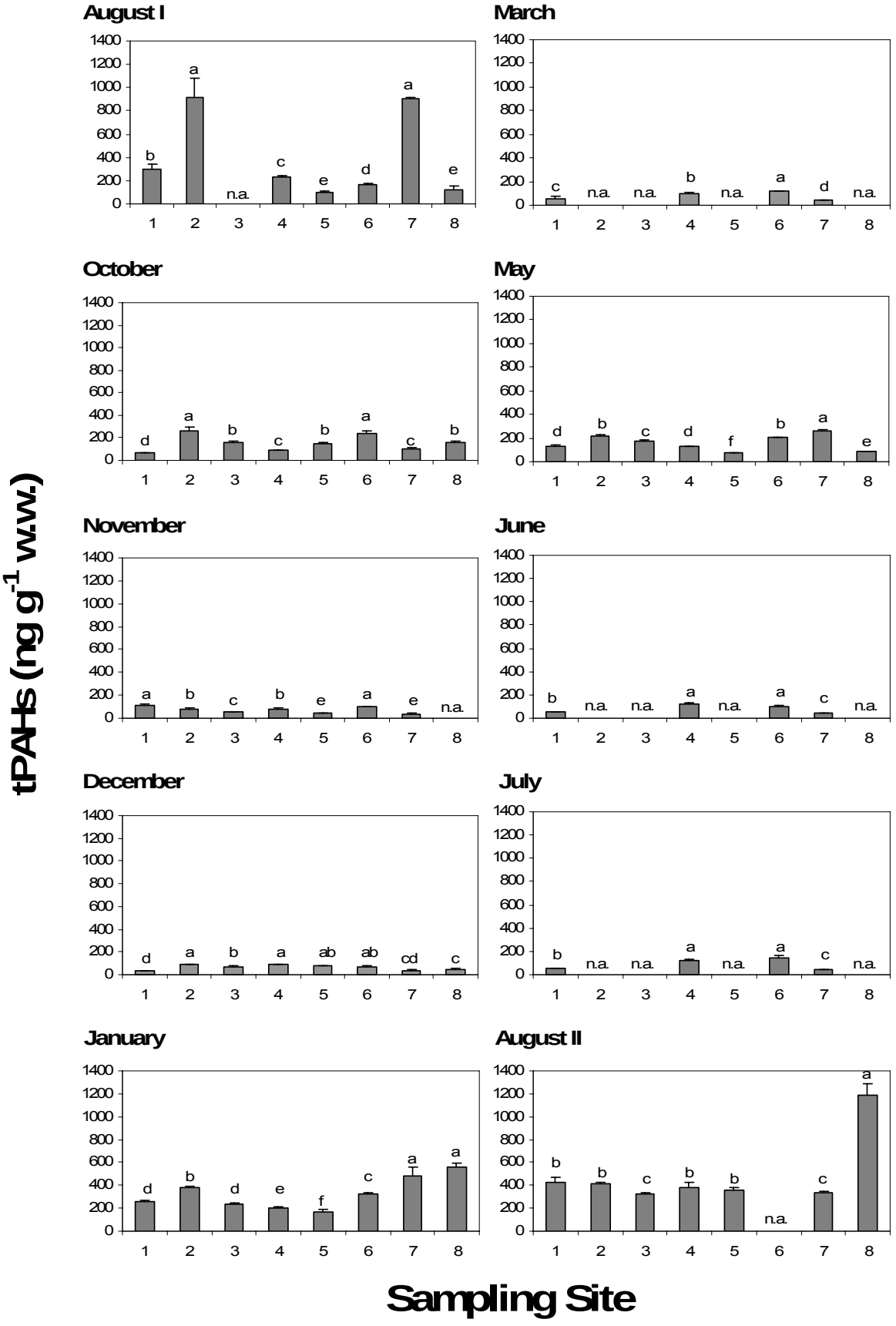


Figure 3.2 – Spatial and seasonal variation of tPAH concentrations (ng g^{-1} w.w.) in clam whole soft tissues. Bars labelled with the same letter are not statistically different (p<0.05) (n.a. – data not available).

Spatial variation of PAHs in clams whole soft tissues were observed monthly (p<0.05), but when the whole data set was considered, no significant differences exist between sites (p>0.05).

Seasonally, the highest tPAH concentrations were in clams collected in August II (p<0.05). The lowest tPAH concentrations were in clams collected in November, December, March, June and July.

In August I (Aug I), tPAH concentrations in clam tissues ranged between 96.2±9.1 and 914±170 ng g^{-1} w.w. The highest tPAH concentrations were in clams from sites 2 and 7, while the lowest tPAH were in those from sites 5 and 8 (p<0.05) (Figure 3.2). Benzo[*a*]anthracene (BaA) (23.8±4.0 – 275±71 ng g^{-1} w.w.), chrysene (Ch) (13.5±2.8 – 121±38 ng g^{-1} w.w.) and fluoranthene (Fluor) (25.2±3.7 – 110±19 ng g^{-1} w.w.) were generally the most abundant PAHs in clam tissues. The spatial distribution pattern of these individual PAHs was similar to that of the tPAH. An exception was in clams from site 4, where the most abundant individual PAH was acenaphthene (Ac) (83.4±17.2 ng g^{-1} w.w.). Figure 3.3, shows the percentage of the individual PAH distribution by aromatic ring numbers in clam whole soft tissues. The individual PAH distribution in clam tissues was dominated by the 4 ring PAHs at all sites (50 – 80%) followed by the 2+3 ring PAHs (12 – 48%). An exception is at site 2, where clams were enriched in 5 and 6 rings PAHs relatively to the lower molecular weight PAHs (28 and 11% respectively).

In October, clam tPAH concentrations ranged from 73.7±0.0 to 263±28 ng g^{-1} w.w. being significantly lower than in Aug I (p<0.05). tPAH concentrations in clams from site 2 were still the highest along with those from site 6, and the lowest were in clams from site 1 (Figure 3.2). The individual PAH distribution in clam tissues was no longer dominated by the 4 ring PAHs. The most abundant PAHs were acenaphthylene (Ace) (3.03±0.57 – 58.5±17.0 ng g^{-1} w.w.) and acenaphthene (11.3±3.1 – 120±16 ng g^{-1} w.w.) in clams from sites 1 to 4, and

phenanthrene (P) (5.22±0.55 – 64.6±7.3 ng g^{-1} w.w.) and benzo[*a*]anthracene (15.8±0.4 – 58.7±11.5 ng g^{-1} w.w.) in those from sites 5 to 8.

In terms of aromatic ring number (Figure 3.2), the 2+3 ring PAHs were dominant in *R. decussatus* from sites 1 to 4 and 7 (46 – 73%), while the 4 ring PAHs were dominant in those from the other sites (52 – 68%).

In November and December, the decreasing tendency in tPAH concentrations in the clam whole soft tissues continued, ranging from 36.9±5.2 to 112±15 ng g^{-1} w.w. in November and from 30.4±3.8 to 85.2±9.2 ng g^{-1} w.w. in December, when the lowest tPAH concentrations occurred (Table A4 and Figure 3.2). In November the highest tPAH concentrations were in clams from sites 1 and 6 and the lowest in those from sites 5 and 7 (p<0.05). In December, tPAH concentrations in clam tissues were less variable, being highest in clams from sites 2 to 6 and lowest in clams from site 1 (p<0.05) (Figure 3.2). In November, the most abundant individual PAHs were acenaphthene (0.59±0.02 – 50.4±13.0 ng g^{-1} w.w.), whose spatial variation was similar to that of tPAH; fluoranthene (4.59±0.89 – 16.1±3.4 ng g^{-1} w.w.), which was highest in clams from sites 3 and 4 and lowest at site 5; and benzo[*a*]anthracene (10.6±1.1 – 25.1±5.2 ng g^{-1} w.w.). In December, the most abundant individual PAH was benzo[*a*]anthracene (8.49±0.43 – 24.4±2.5 ng g^{-1} w.w.), being highest at clams from site 3 and lowest in clams from site 1 (p<0.05). This means that the 2+3 rings PAHs no longer dominated the distribution of the individual PAHs in clam tissues (Figure 3.3) and, like in Aug I, the 4 aromatic ring PAHs were the most abundant (57 – 77%), except for site 1 in November, where the 2+3 rings still predominated (59%). In December, the PAHs distribution pattern was completely dominated by the 4 ring PAHs at all sites (65 – 80%).

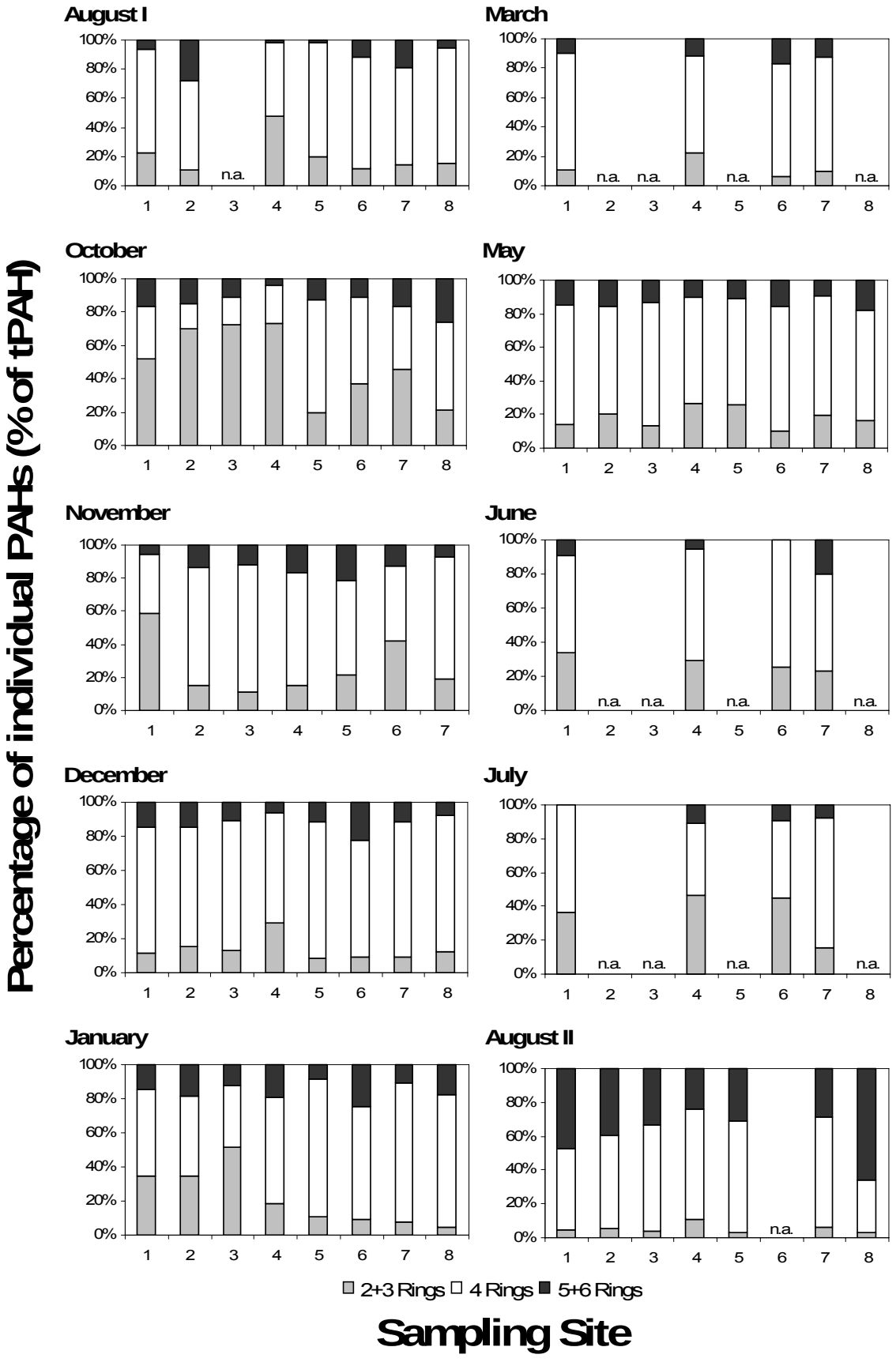


Figure 3.3 – Distribution pattern of the individual PAHs grouped by number of aromatic rings in the clam whole soft tissues (n.a. – data not available).

The decreasing tendency in tPAH concentrations from October to December, was reversed in January with a significant increase in clam PAH concentrations (p<0.05). tPAH concentrations ranged from 164±21 to 556±34 ng g^{-1} w.w. (Table A4). Significantly higher tPAH concentrations occurred in clams near the only continuous freshwater input to the Ria Formosa lagoon (site 7) (p<0.05). The lowest tPAH concentrations were in clams from site 5 (Figure 3.2). As in the previous months, benzo[*a*]anthracene (20.8±0.2 – 212±11 ng g^{-1} w.w.) was the most abundant individual PAH in clam whole soft tissues except at sites 3 and 7 where acenaphthene (5.30±1.07 – 60.0±4.6 ng g^{-1} w.w.) and chrysene (34.0±1.2 – 142±29 ng g^{-1} w.w.) predominated. In terms of PAH ring number (Figure 3.2), the 4 ring PAHs (47 – 81%) continued to be the most accumulated in clam tissues from all sites except in those from site 3, where the the 2+3 ring PAHs (51%) dominated.

In the following months (March, May, June and July) tPAH concentrations significantly decreased, when compared to January (p<0.05). In March, tPAH concentrations ranged from 44.3±1.7 to 117±6 ng g^{-1} w.w. Benzo[*a*]anthracene (16.4±0.5 – 42.5±3.0 ng g^{-1} w.w.) was still the most abundant PAH at all sites, confirming the dominance of the 4 ring PAHs (66 – 80%) (Figure 3.3). In May, tPAH concentrations in clam tissues were significantly higher than in March (p<0.05), ranging from 76.0±3.3 to 257±17 ng g^{-1} w.w., however, the highest tPAH concentrations were in clams from site 7 and the lowest in those from site 5 (p<0.05) (Figure 3.2). As in the previous month, the most accumulated individual PAH in clam tissues was benzo[*a*]anthracene (22.3±2.7 – 80.1±10.4 ng g^{-1} w.w.) and the 4 ring PAHs (64 – 74%) continued to predominate at all sites (Figure 3.3).

In June and July clams had similar tPAH concentrations (p>0.05) and significantly lower than in May (p<0.05). In these months, tPAH concentrations in clam tissues ranged from 42.6±2.3 to 119±10 ng g^{-1} w.w. in June and from 45.0±3.8 to 145±19 ng g^{-1} w.w. in July. The most abundant PAH in June was benzo[*a*]anthracene (10.7±0.8 – 39.7±4.7 ng g^{-1}

w.w.) and, in July, acenaphthene (0.53±0.03 – 49.4±10.9 ng g^{-1} w.w.) along with benzo[*a*]anthracene (11.5±0.2 – 33.4±1.3 ng g^{-1} w.w.). Analysing the individual PAHs distribution pattern, the predominance of the 4 ring PAHs was maintained (43 – 76%).

tPAH concentrations in the clam whole soft tissues increased once more in August II (325±9 – 1191±92 ng g^{-1} w.w.) being the highest of the whole period (Table A4; Figure 3.2). However, contrary to August I, in August II, the highest tPAH concentrations were in clams from site 8 and the lowest in those from sites 3 and 7 (p<0.05). The most abundant individual PAHs were benzo[*a*]anthracene (72.8±4.4 – 149±16 ng g^{-1} w.w.) and chrysene (66.3±3.7 – 137±15 ng g^{-1} w.w.) at all sites, except at site 8, where the concentration of dibenzo[*a,h*]anthracene was the highest (312±44 ng g^{-1} w.w.). The distribution of the individual PAHs in clam tissues was significantly different from the previous months (p<0.05). The 4 ring PAHs were still the most abundant hydrocarbons (31 – 66%), in most sites, followed by the 5+6 ring PAHs (24 – 66%) and the 2+3 ring PAHs (3 – 11%).

Figure 3.4 presents the monthly average of total lipid content of clam tissues that ranged from 0.61±0.26% to 1.21±0.26%.

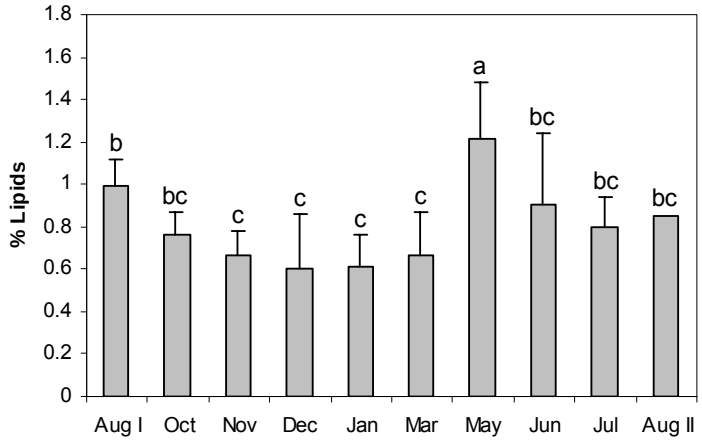


Figure 3.4 – Monthly mean lipid content (%) in the clam whole soft tissues from all sites. Error bars are standard deviation. Bars labelled with the same letter are not statistically different (p<0.05).

Like for tPAH concentrations, there were no significant differences between sites (p<0.05) and therefore, the most important variation was seasonal. The maximum lipid content was in spring (May) and the minimum in winter (November through March) (p<0.05). Therefore, the tPAH accumulated in the whole soft tissues was not directly related with their lipid content (p>0.05).

To study the spatial and seasonal variation of the clam individual PAHs, Principal Component Analysis (PCA) was performed on individual PAHs accumulated in clam tissues, as a proportion of tPAH. Figure 3.5 presents the loadings diagram for the centred log transformed data (see Section 2.2 for further details). Although the explained percentages were low (PC 1 explains 33.5% and PC 2 explains 24.7%), they were statistically significant.

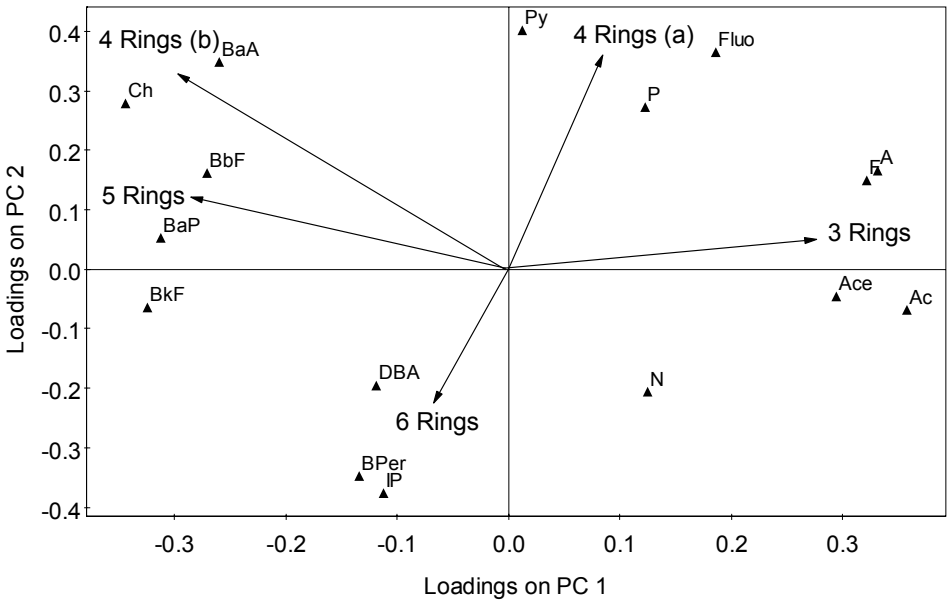


Figure 3.5 – PCA of the individual PAH as a proportion of the tPAH, in the whole soft tissue of the clams *Ruditapes decussatus*, showing the loadings on PC1 and PC2. PC1 represents 33.5% and PC2 24.7% of the total variance in the data, both being significant (p<0.05; n = 65). Data was centred and log transformed.

In this diagram, similarly to the sediments (Figure 2.8), the individual PAHs in clam tissues were distributed by aromatic ring number signed by vectors leading to five different groups. The first vector points to the 3 aromatic rings group (acenaphthylene, acenaphthene,

fluorene and anthracene). The second vector, points to the group "4 rings (a)" which includes fluoranthene, pyrene and also the 3-aromatic ring phenanthrene. The third vector points to the other 4 aromatic rings benzo[*a*]anthracene and chrysene. These are the more relevant hydrocarbons in this data set, since they present the highest loadings on both PC 1 and PC 2. The fourth and fifth vectors point to the 5 and 6 aromatic rings group, respectively.

This variable arrangement resulted in a scores plot where the similarity and grouping of the samples were indicated. Figure 3.6 presents a plot of the scores samples shown by month with the loading vectors overlaid. In this plot, samples are less separated from each other, compared to the sediment plot (Figure 2.9). In fact, most of the samples are close to each other, in the centre of the plot, indicating that the individual PAHs distribution is similar in most of the samples, and consists of a mixture of several PAHs, without any enrichment in a particular one.

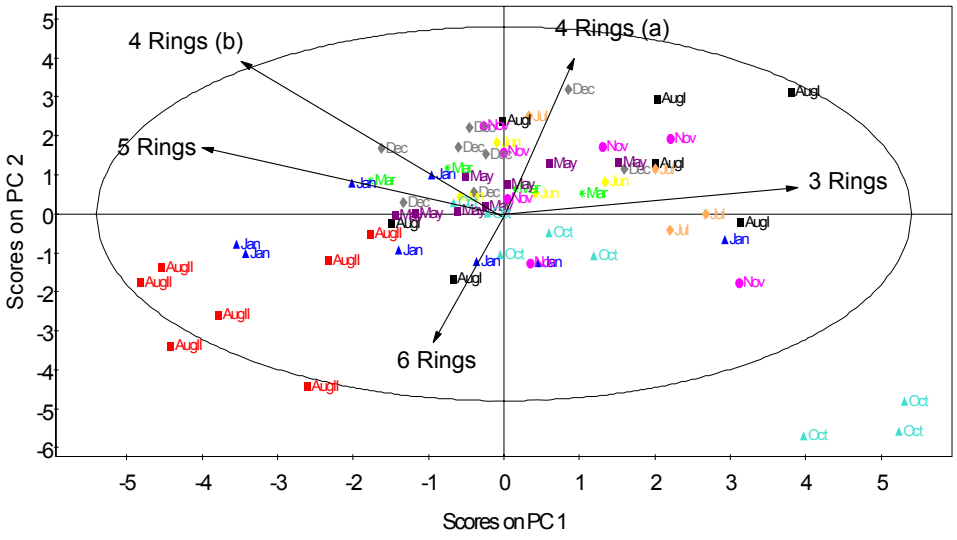


Figure 3.6 – PCA of the individual PAH as a proportion of the tPAH, in the clams *Ruditapes decussatus*, showing the data scores labelled by month.

Some of the October samples, however, are located in the lower right quadrant of the plot, outside the Hotelling's T^2 circle (see section 2.2). This indicates that, in these samples, the

PAHs distribution was enriched in the lower molecular weight hydrocarbons and depleted of the 4 ring PAHs, benzo[*a*]anthracene and chrysene as observed in Figure 3.2 and Table A4 in Annexe. In a similar way, the August II samples, that have the highest tPAH concentrations of all the clams sampled, loaded negatively on both PC1 and PC2, indicating that the 5-ring and larger (6-rings) hydrocarbons were dominant. In fact, in August II, the concentration of dibenzo[*a,h*]anthracene was the highest, specially at site 8. Similar conclusions had been drawn from Figure 3.2, that is, a predominance of the 2+3 ring PAHs in some October samples and higher amounts of the 5+6 ring PAHs in the August II samples.

When the data were labelled according to their site number (Figure 3.7), no spatial associations are apparent, indicating the dominance of temporal variability over the spatial variability.

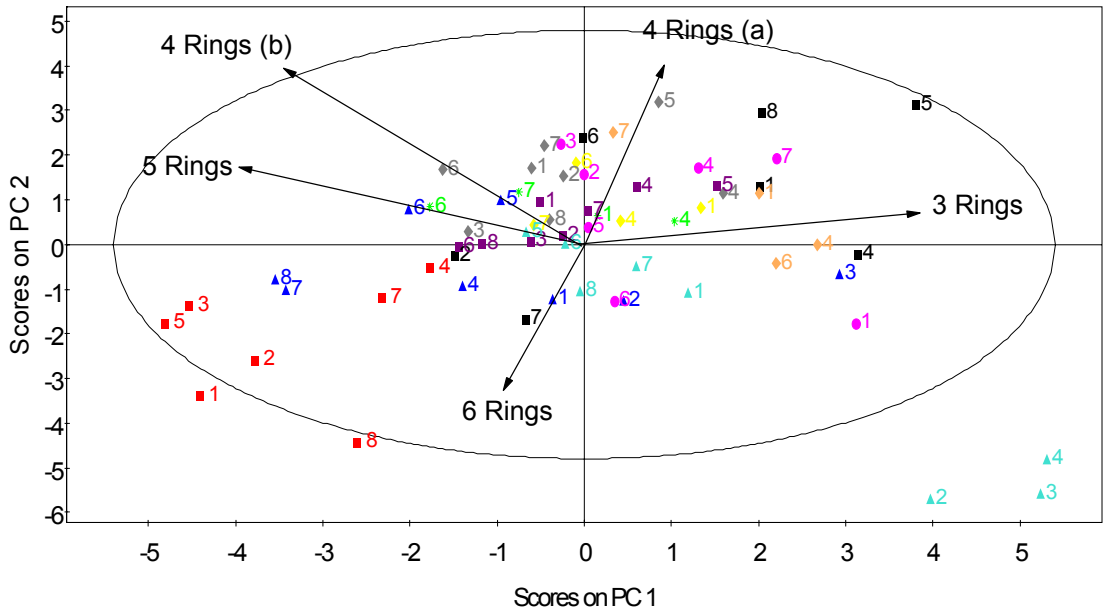


Figure 3.7 – PCA of the individual PAHs as a proportion of the tPAH accumulated in the clams *Ruditapes decussatus*, showing the data scores labelled by site number. Different colours were attributed to the sampling months as in Figure 3.6.

This means that there is a change in PAHs signature in the clams over time, common to all sites, similarly to the observed for the sediments.

The variation of PAH signature in clam tissues with time is probably the reflection of a similar variation in the source term. In this case, the analysis of specific ratios as the phenanthrene/anthracene (P/A), the fluoranthene/pyrene (Fluo/Py) and the low over high molecular weight PAH ratios may help to assess these origins in clam tissues (Table 3.3).

Table 3.3 – Phenanthrene/Anthracene (P/A), Fluoranthene/Pyrene (Fluo/Py) and Low/High molecular weight PAHs ratios in clam whole soft tissues.

	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8
P/A Ratio								
Aug I	5.40	4.75		6.36	5.86	7.35	6.74	7.00
Oct	7.69	3.23	2.72	1.66	15.3	77.1	40.6	1.88
Nov	4.17	6.32	7.30	8.05	8.56	7.95	6.90	
Dec	8.98	8.22	11.7	6.06	8.25	8.50	8.83	0.79
Jan	8.31	1.10	8.58	9.00	6.58	10.7	7.32	9.40
Mar	0.75			7.00		17.1	4.64	
May	7.39	8.58	15.2	13.3	11.0	7.88	11.5	18.3
Jun	7.20			4.92		10.1	16.7	
Jul	9.15			4.92		8.38	10.9	
Aug II	31.3	16.7	14.2	6.81	17.1		11.0	1.35
Fluo/Py Ratio								
Aug I	1.81	0.49		1.80	2.02	1.27	0.80	1.40
Oct		1.74	2.26	3.97	0.99	1.02	0.71	1.26
Nov	1.46	1.28	1.42	2.46	1.15	0.94	1.40	
Dec	1.42	1.08	0.87	0.76	1.46	1.21	1.58	1.68
Jan	0.88	0.28	1.55	1.76	0.97	1.17	1.11	1.22
Mar	2.12			2.57		1.70	1.04	
May	1.59	1.41	0.88	0.97	1.32	2.02	0.36	0.89
Jun	0.74			1.04		1.33	1.17	
Jul	0.72			1.74		1.58	0.46	
Aug II	0.66	0.96	0.83	0.75	0.85		0.90	0.73
Low/High Ratio								
Aug I	0.29	0.12		0.91	0.25	0.13	0.42	0.18
Oct	1.07	2.35	—	2.69	0.25	0.59	0.84	0.27
Nov	1.42	0.17	—	1.69	0.27	0.72	0.24	
Dec	0.13	0.18	—	0.09	0.41	0.10	0.11	0.14
Jan	0.52	0.53	—	0.23	0.12	0.10	0.08	0.05
Mar	0.12			0.29		0.06	0.11	
May	0.16	0.26	—	0.36	0.34	0.13	0.24	0.20
Jun	0.51			0.62		0.28	0.30	
Jul	0.58			0.86		0.81	0.17	
Aug II	0.05	0.05	0.04	0.12	0.04		0.07	0.03

Low = 2+3 aromatic ring PAHs; High = 4+ aromatic ring PAHs.

The P/A ratios in clam tissues were lower than 10 in most cases, and higher than 25 in only three of the samples, which is indicative of petroleum contamination. These were in clams from sites 6 and 7 in October, which had P/A ratios of 77.1 and 40.6 (respectively) and in clams from site 1 in August II (31.3). These sites are relatively close to fishing ports and recreational marinas (sites 6 and 7) and site 1 is in the main entrance of commercial vessels in the Ria Formosa.The Fluo/Py ratio (Table 3.3) ranged between 0.28 and 3.97. There was no evident tendency in this ratio, with all sites and months presenting large variability in Fluo/Py ratios. An exception occurred in August II, which had ratio always lower than 1, indicative of petrogenic rather than pyrolytic PAHs source.

The ratio between the low molecular weight (2+3 aromatic rings) and the high molecular weight (4+5+6 aromatic rings) hydrocarbons in clams whole soft tissues (Table 3.3) was generally lower than 1 confirming earlier results on individual PAHs in which the 4+ aromatic ring hydrocarbons were the most abundant in clam tissues. Exceptions occurred only in October (sites 1, 2 and 4) and November (sites 1 and 4). The lowest ratio was in winter, spring (from December to May) and summer (August II), which presented a signature enriched in higher molecular weight PAHs.

Similarly to the sediment samples, PLS analysis was used to clarify the origin of these PAHs in clam tissues. The nineteen PAH signatures described in section 2.2 were used. Each signature was fitted to the clam whole soft tissue PAH data. The amount of variance explained by the most relevant signatures is presented in Figure 3.8 as stacked bars.

In several samples, less than 20% of the total variance in the clam data is explained and therefore these signatures are insufficient to describe all the variance in the data. It is possible that the right signature is not among the nineteen used. However, there is also the possibility that PAH signatures may have been altered by both uptake and metabolisation of these contaminants by the clams. This could be confirmed if benzo[*e*]pyrene (BeP)

concentrations were known since ratios of (BeP) and benzo[*a*]pyrene (BaP) lower than 1 indicate PAHs biotransformation (Geffard *et al.*, 2003). Some samples, occurring mainly in October, June and July were not explained by any of the signatures used. A reasonable amount of samples, however, had explained variances between 20 and 100%. In these cases, the major sources from the signatures used are signatures C, D, F, H, O, P and S (tar). Signature C is a background PAHs signature found in offshore samples (100 to 1000m from shore) being characterized by a much higher proportion of the 2+3 aromatic ring PAHs. This signature was found mainly in August II samples.

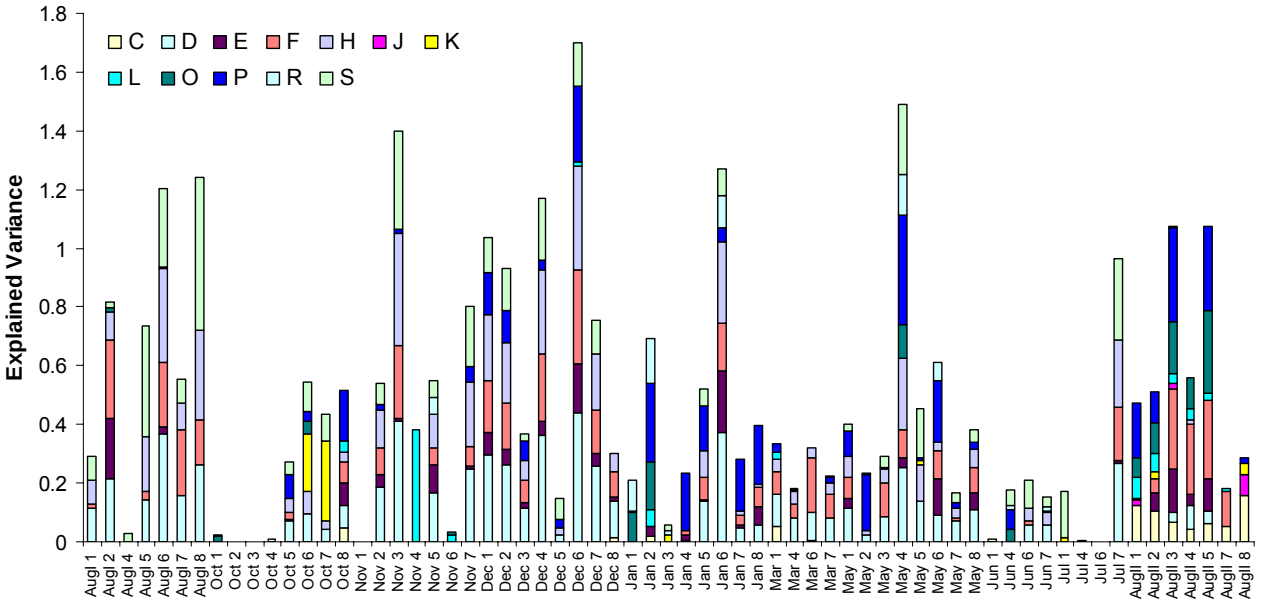


Figure 3.8 – Partial Least Squares analyses of the PAH signatures in the clams whole soft tissues (15 *observations*; 92 *variables*). Nineteen different PAH signatures, taken from the literature were used to develop the X-block (see Section 2.2 for further details).

Signature D, however, was found in almost all samples. This is a PAH signature of harbour sediment, presenting high proportions of the 4+5+6 aromatic ring PAHs and almost no 2+3 aromatic ring PAHs. It probably represents the influence of ships traffic. Signature F indicates the influence of household soot, that is, combustion products generated in domestic activities. This signature was common to most samples. Signature H is also a combustion

signature, however it was greatest during the winter months, which may be expected if wood materials are used for domestic heating. There are also some enhanced values for this signature in the summer that may be associated with charcoal burning or forest fires, both common in the summer. Signature J is characteristic of diesel oil contamination. It was found in just a few samples in July (site 7) and August II (sites 1 and 8). This type of signature may be related to boats traffic and port activities. Similarly, signature K was found in a small number of samples. However it explained high amounts of variance in two October samples (sites 6 and 7). This signature may indicate the presence of diesel soot generated by boats. Signature L, although present in just a few samples, is an unmodified crude oil signature. It explained a high amount of variance in the sample Nov 4, being the only relevant signature for this sample and for some of the August II samples (sites 1 to 7). Signatures O and P indicate the presence of, respectively, moderately and heavily weathered oil. Although signature O was just found in two January samples (sites 1 and 2) and in most August II samples (sites 1 to 5), signature P was found in the majority of the samples. These signatures may indicate the presence of environmentally aged PAH contamination or the result of the clam metabolism, which modified the oil signature. The tar signature (S) appears in several sites scattered throughout the year and is not readily explainable. This signature may be associated to road run-off of asphalt type materials, which are washed out of the catchments and enter the lagoon during periods of abundant rain. This is unlikely to be the case in August as rainfall is very low at this period. It is possible that the source of this type of signature is being confused with other related or similar signatures, as creosote, which has a similar PAHs distribution pattern. Creosote is used as a wood preservative and though its use was banned in 1999 (DL 446/99) its use in pilings and boats could explain its scattered appearance in the Ria Formosa (Page *et al.*, 1999).

To assess the possible implications for human consumption of these bivalves, the amount of BaP equivalents (BaP eq) was calculated for each sample (Figure 3.9). The majority of the samples had a BaP eq<10 ng g^{-1} w.w. and could be considered safe for human consumption. A few samples from Oct, Jan, May and Aug II exhibited BaP eq>10 ng g^{-1} but less than 45 ng g^{-1} w.w. For these cases, further studies are needed, although still admissible for human consumption.

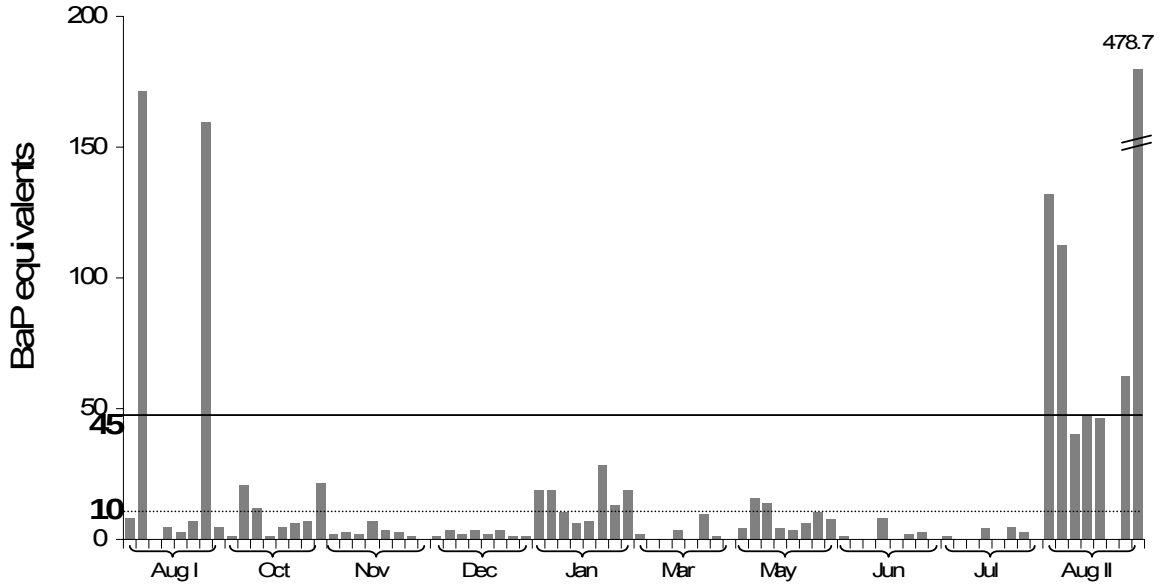


Figure 3.9 – Benzo[*a*]pyrene equivalents obtained for PAHs in clams tissues. The broken line represents the safety level for human consumption of shellfish (10 ng g^{-1} w.w.) and the upper line the maximum allowed (45 ng g^{-1} w.w.).

However, sites 2 and 7 (in Aug I) and sites 1, 2, 7 and 8 (in Aug II) had BaP eq higher than 45 ng g^{-1} w.w. and therefore could be unsafe for human consumption. These samples corresponded to the highest PAH concentrations where the 5 and 6 aromatic ring PAHs were a considerable proportion of the tPAH accumulated by the clams. In clams from site 8 (Aug II) the BaP eq were 10 times higher than the maximum permitted for human consumption by the Oregon Health Division (Gilroy, 2000). It is necessary, however, to consider man's daily

intake of these shellfish. In clams with more than 45 ng g^{-1} w.w. BaP equivalents its consumption should be reduced to less than one meal (250 g) per month (Gilroy, 2000). If clams have less than 10 ng g^{-1} w.w. of BaP equivalents, the consumers are "allowed" more than 1 meal (250 g) of clams per week (Gilroy, 2000).

3.3.2. Relationship between PAHs concentrations in clam whole soft tissues and sediments

To assess if PAH concentrations in clam tissues are related to those in the sediments, PAH content was compared in both compartments. Normal distribution of the data was obtained with a log transformation. Figure 3.10 shows the relationship of the log transformed tPAH content in the sediments against the log transformed tPAH content of clam tissues. Using the January through August II data, no significant relationship exist between tPAH concentrations in these compartments (p>0.05). However, this is probably due to the August samples, where PAH concentrations were higher in clam tissues but not in the sediments, probably indicating a different contamination source in this period. When the August data is not included, a significant positive relationship exist between these two variables (log $tPAH_{Clams}$ = 1.81 + 0.42 log $tPAH_{Sed}$; r = 0.640; n = 28; p<0.001).

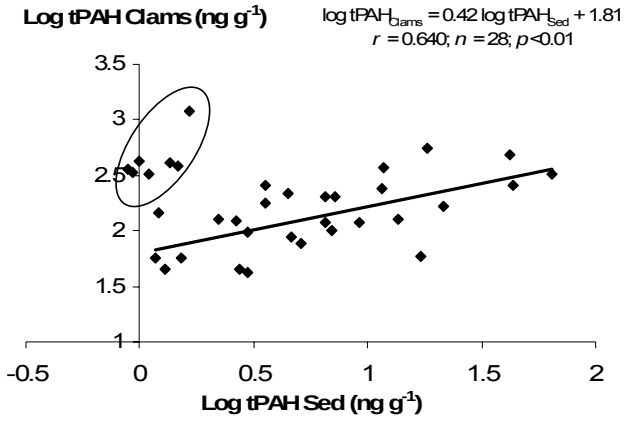


Figure 3.10 – Relationship between log tPAH concentrations in the sediments versus log tPAH concentrations in the clam whole tissues. The circle indicates the August II data (not included in the relationship).

Considering the individual PAHs, similar results were obtained. That is, the concentration of each individual PAH in the clam tissues is not always related with the same individual PAH in the sediments. Exceptions were observed for anthracene and benzo[*b*]fluoranthene; positive significant relationships exist for these compounds in both compartments (log A_{Clams} = 0.38 + 0.40 log A_{Sed}; r = 0.352; n = 35; p<0.05; log BbF_{Clams} = 1.28 + 0.36 log BbF_{Sed}; r = 0.698; n = 14; p<0.05), although with significant lower slopes than the relationship between tPAH concentrations in both compartments. Again, if the August samples were not included, the significance of these relationships increased.

If tPAH concentrations in the sediments are normalised with the organic carbon content and in the clams whole tissues with their lipid percentage, no relationship between tPAH concentrations in both compartments exist. Still, if the August data is not considered, a positive linear relationship exist between both variables (log $tPAH_{Clams}$/%lip = 1.94 + 0.49 log $tPAH_{Sed}$/%OC; r = 0.521; n = 26; p<0.01), similar to the relationship between the not normalised tPAH concentrations in the sediments versus the clams.

To quantify the PAHs accumulation in clam tissues from the sediments, biota sediment accumulation factors (BSAF) were calculated for each individual PAH (see section 1.1.5 for definition). The results are presented in Annexe (Table A5). Generally, BSAF increased with the increase in the PAHs molecular weight and K_{ow} values.

It is usually considered that the bioavailable PAHs are preferentially accumulated in the lipidic tissues of the organisms. In this study, although no relationship occurred between tPAH concentrations in clams tissues and its lipid content, a significant positive linear relationship exists between log BSAF values and log K_{ow} for each individual PAH (log BSAF = - 0.87 + 0.50 log K_{ow}; r = 0.554; n = 277; p<0.01). Analyzing this relationship on a monthly basis, different slopes were obtained (Figure 3.11).

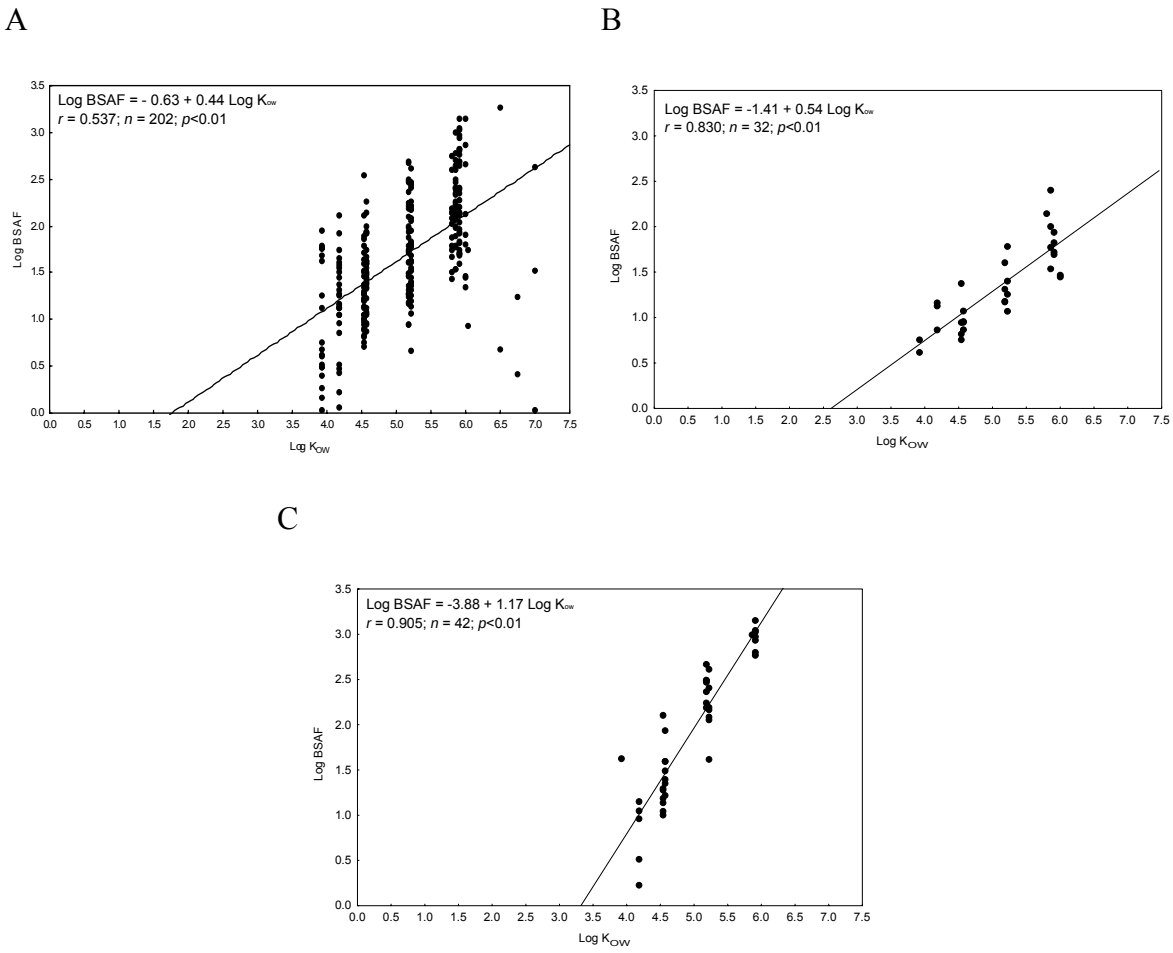


Figure 3.11 – Relationships between log BSAF and log K_{ow} in the different sampling months. A – Jan, Mar, May and Jul; B – Jun; C – Aug.

The slopes in January, March, May and July were similar (p>0.05). Thus, a common slope was calculated for these months (log BSAF = - 0.63 + 0.44 log K_{ow}; r = 0.537; n = 202; p<0.01) (Figure 3.11 A). In June, the slope was significantly higher than in the previous months (0.54) and in August even higher (1.17) (Figure 3.11 B and C). That is, in June (Figure 3.11B) and August (Figure 3.11 C), clams preferentially accumulated the high molecular weight hydrocarbons relative to the low molecular weight PAHs, which caused the slope to increase.

So far, the relationship between the PAH accumulated in the clam tissues and in the sediments has been analysed in terms of PAH concentrations. This relationship may be

further analysed using the proportion of each individual PAH in the tPAH concentration. This study was performed using Partial Least Squares (PLS) analysis.

The sediment data were used to generate a signature (X-block) in order to explain the variance that exists within the clam PAH data (Y-block). Figure 3.12 shows the amount of variance explained in each of the clam sites and month (grey bars – R^2) by the sediments and also the fraction of the total variance of the Y-block that can be predicted by each extracted component (white bars – Q^2).

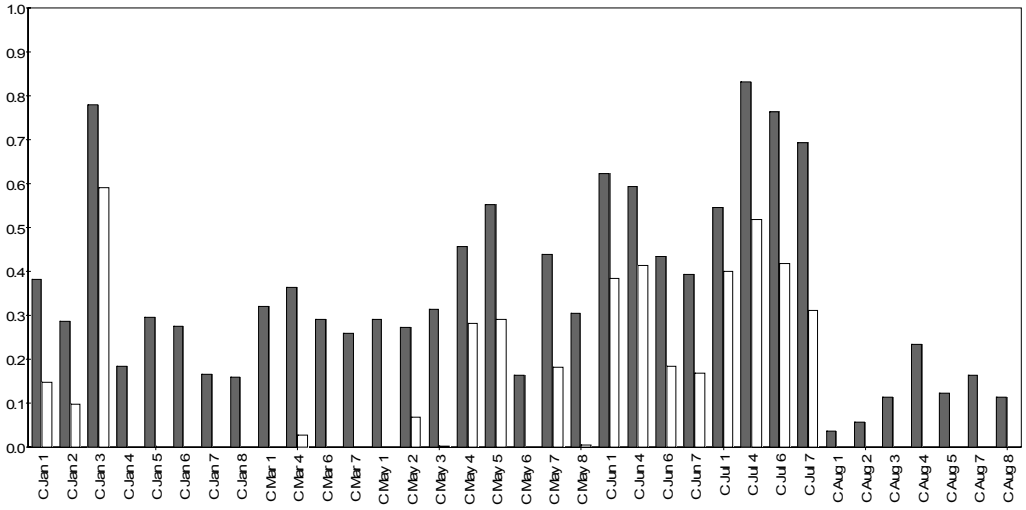


Figure 3.12 – Partial Least Squares analysis using the sediments PAHs signature as X-block (15 *observations*; 71 *variables*). Bars show the amount of variance within the clam data explained (grey) and predicted (white) by the sediments. Data were not transformed.

From Figure 3.12, it can be seen that, in many instances the sediment data do not explain much of the variance within the clams (the grey bars are low), especially in August confirming earlier results (Figure 3.10). Additionally, PAHs in the clam samples cannot be or are poorly predicted by the PAH signature in the sediments. In these cases the white bars are zero. However, in May, June and July some of the variance in the clam tissues seems to be predicted by the sediment data. These values reach approximately 50% at site 4 in July. The January data exhibits also a small amount of predicted variance. The largest value, however,

was at site 3, in January, where the PAHs signature in the sediments can predict almost 60% of the variance in the clams.

Having done PLS, a plot of the loadings on Principal Component 1 in the X-block (T1) was plotted against the loadings on Principal Component 1 within the Y-block data (U1) in order to determine if the compounds used are similar and appropriate for signature analysis (Figure 3.13).

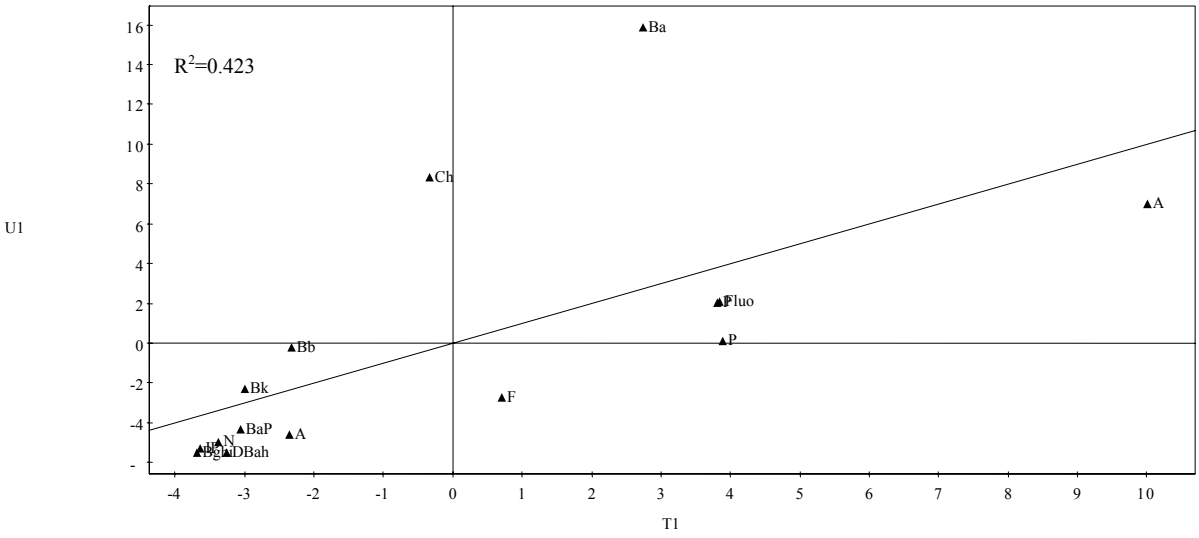


Figure 3.13 – PLS XY score plots for Principal Component 1.

In this plot, the straight line indicates the one to one relationship between loadings in the X-block and in the Y-block. R^2 is a measurement of the variance explained in the T1/U1 plot by the one to one relationship. If the PC 1 of the X-block (T1) explains most of the variance in the Y-block (U1), the R^2 value will be close to 1. Analysing this figure, it is evident that two PAHs are significantly different within these two blocks, since they are distant to the line. These are chrysene (Chr) and benzo[*a*]anthracene (BaA), which load significantly more positive on U1 than they do on T1. This means that clams are more influenced by these two four ring compounds than the sediments in which they are buried. In

a plot like this, the compounds that are furthest away from the origin (0;0) also have the greatest weight and therefore the greatest effect on the final signature. Thus, acenaphthene (Ac) is the most relevant individual PAH in the final signature, although for both sediments and clams.

Since Chr and BaA were obviously different between the X-block and Y-block data, these two compounds were removed from the data set and the PLS analysis recomputed. The U1 versus T1 plot (Figure 3.14) now has a much higher R^2 value (0.822).

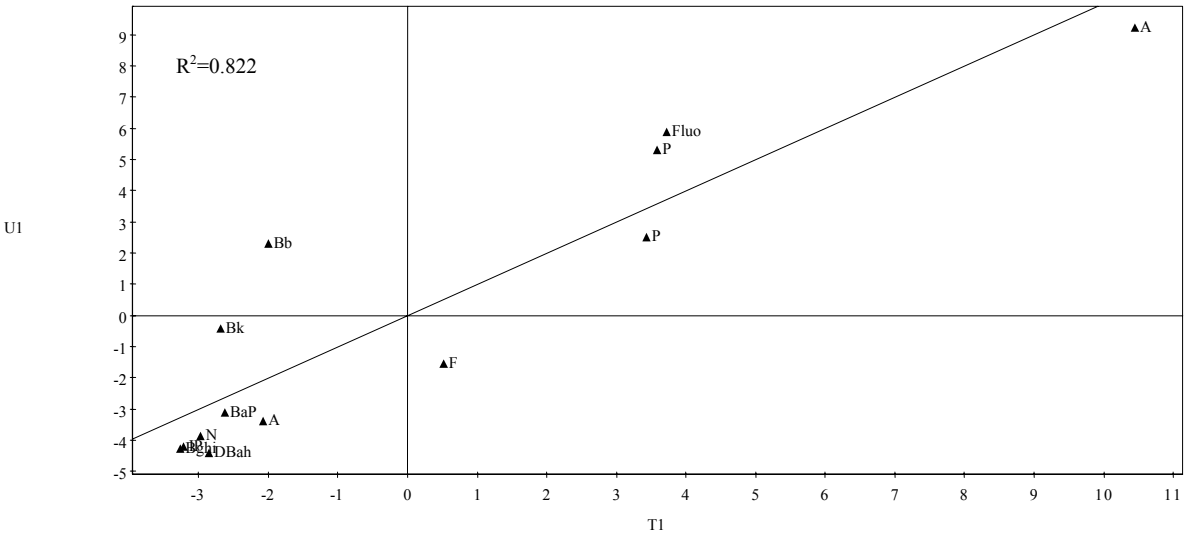


Figure 3.14 – PLS XY score plots for Principal Component 1 excluding Ch and BaA data.

This reinforces the result seen in the previous diagram where Chr and BaA were significantly enriched in the clams tissues and therefore distorting the signature. With these two PAHs removed a much greater proportion of the variance in the clam data can now be explained and predicted by the sediments. These values reach almost 90% in the June and July, although they are still lower in March and May. As mentioned before (see section 3.3.1), PAHs concentrations were lower in these months and BaA was the most abundant at all sites. The August clam samples, however, are still not explained nor predicted by the sediment data.

As well as using the sediment data to try to explain the PAHs variance within the clam tissues, it is possible to use the clam PAHs data to try to explain the signatures seen within the sediments. Therefore, the clam data were used as the X-block and the sediment data as the Y-block. All compounds were used, without any transformation. The result is presented in Figure 3.15. The most obvious feature in comparison to the previous data is that the explainable variance is much higher.

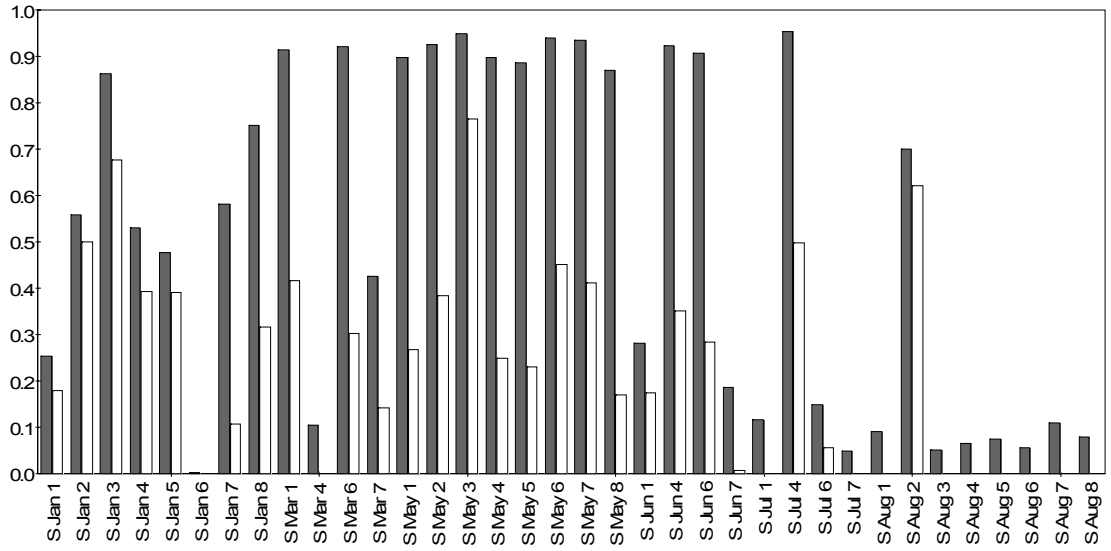


Figure 3.15 – Partial Least Squares analysis using the clams PAH signature as X-block and sediments as Y-block (15 *observations*; 71 *variables*). Bars show the amount of variance within the sediments data explained (grey) and predicted (white) by the clams. Data were not transformed.

The Chr and BaA were also removed in this second analysis as these two compounds also exhibited a different signature within the clams compared to the sediments. That caused the explainable variance to increase, but only in those sites where some of the variance was already explained.

The same was true for the predictable fraction of the variance. For example, in the August data those sites that had no predicted variance with chrysene and benzo[*a*]anthracene present still have no predictable variance with those compounds removed.

These results reinforce the difference between the PAHs concentration of clam tissues and sediments.

3.4. DISCUSSION

This is the first study on PAH concentrations in the bivalves from the Ria Formosa lagoon. The mean tPAH concentrations in the clams from most sites in the Ria Formosa were similar to those of the clams *Tapes philippinarum* and *Venus gallina* from different European locations (Binelli & Provini, 2003) and in *Tapes semidecussata* from the Galicia Coast (Porte *et al.*, 2000) (see Table 1.4 in Chapter 1). There were, however, some isolated but rather high PAH concentrations in *R. decussatus* tissues in the summer (at sites 2, 7 and 8), similar to those found in clams from areas of high petroleum contamination (Porte *et al.*, 2000).

The spatial and seasonal variation of PAH concentrations in the clam tissues revealed that seasonal factors prevailed over spatial ones. tPAH concentrations in summer (August I and II) and winter (January) were higher than in the other months. However, there was no particular site with consistently higher or lower tPAH concentrations (Figure 3.2).

The lipid content of *R. decussatus* was similar to that reported by Ferreira and Vale (1998) for the same species (Ferreira & Vale, 1998). The highest values were in May (Figure 3.4) probably associated with the reproductive cycle. Similarly, *R. decussatus* from the Mundaka Estuary in Urdabai (Basque Country, North Spain) presented increased somatic growth in the spring associated to a period of fastening and growth, prior to spawning (Urrutia *et al.*, 1999). Tissues with higher lipid content generally accumulate PAHs to a greater extent (Livingstone, 1998). However, maximum PAH concentrations in the clam tissues (in August) did not correspond to a maximum in tissue lipid content. In fact, no relationship exists between lipids and tPAH concentrations in clam tissues. Instead, PAHs

seasonal variation in clam tissues is probably related to the environmental PAH load, which is associated to the seasonality in PAH sources to the Ria Formosa lagoon. Similarly, no relationship between lipid content and PAH concentrations were found in *Mytilus sp.* from the Arcachon Bay (Devier *et al.*, 2005) or PCB and DDT concentrations in several dwelling-bivalves, including *R. philippinarum* (Thompson *et al.*, 1998).

In order to interpret the PAHs seasonality, an approach similar to the sediments was used. As observed for the sediments, Principal Components Analysis indicated that the variation in source changed with season but was common to all sites (Figure 3.6 and 3.7). The August I data was characterized by higher content of 2 to 4 aromatic ring PAHs, suggesting a mixed petrogenic and pyrolytic source. On the contrary, August II samples had significant higher amounts of PAHs and especially of 5+6 aromatic ring PAHs, than the rest of the samples, which indicates a definite pyrolytic source. Samples from December, March, May and June presented a mixed composition of 2+3 and 4 aromatic ring PAHs and thus, like August I, a mixed petrogenic and pyrolytic source. The October samples were split between the other two groups, one of them included sites 2, 3 and 4, presenting a definite petrogenic signature, with much higher proportions of the 2+3 aromatic rings. In the January samples PAH sources were different at each site. Nonetheless, since each PAH source produces a specific fingerprint (Baumard *et al.*, 1999a), there is a change in source of PAHs in the Ria Formosa lagoon through out the year. It also indicates that the PAH sources ranged from a petrogenic to a pyrolytic source and a mixture of both, except in January.

The PAH source allocation assessed by analysing individual PAH ratios as the phenanthrene/anthracene (P/A), fluoranthene/pyrene (Fluo/Py) and 2+3 aromatic rings/4+5+6 aromatic rings (Low/High molecular weight) (Table 3.3) (see Chapter 1 for further detail) revealed that these ratios did not change spatially nor seasonally, which was not consistent with the sources allocation by the PAH distribution pattern. This is probably a reflection of

the factors governing the accumulation of contaminants by these suspension-feeder bivalves. As hydrophobic contaminants, an inverse relationship exists between the PAH partition coefficient and its water solubility (Neff *et al.*, 1996; de Maagd *et al.*, 1998). Thus, the more soluble hydrocarbons are generally more bioavailable than the less soluble PAHs. For instance, phenanthrene has a K_{ow} 20 times lower and is almost 100 times more water-soluble than anthracene; therefore, it is more bioavailable for accumulation than anthracene. In fact, phenanthrene concentrations in the clam tissues were always higher than anthracene concentrations (Table 3.3). In some cases, however, P/A ratio was higher than 25, which is indicative of petroleum contamination. Moreover, the average P/A ratio was 1.7 times higher for clams than for sediments (Table 2.5). This means that the clam tissues were enriched in phenanthrene and therefore the water pathway was probably more significant. Solubility differences between fluoranthene and pyrene are not so clear, fluoranthene is only 1.8 times more soluble than pyrene, and they have similar K_{ow} values. In fact, Fluo/Py ratios in clams (average 1.28) (Table 3.3) and in sediments (average 1.25) (Table 2.5) were similar. Thus, their bioavailability to clams might be similar (Fluo/Py ratio close to 1). Considering also the differences in the amounts of benzo[*a*]anthracene and chrysene accumulated in clam tissues, the first is almost 4 times more soluble than chrysene and it was generally accumulated in higher concentrations in clam tissues (Table A4 in Annexe).

The PAH source allocation by the PLS approach was unable to clarify the PAH origins in a large number of samples. Most of these occur in October, March, May, June and July (Figure 3.8). In these samples, either the wrong signatures were used or PAHs were differentially uptaked or modified after being accumulated and were no longer identified. Still, some signatures were able to explain a reasonable amount of the variance in most of the samples.

In August I, PLS revealed signatures D, E, F, H and S. These signatures are related to boat traffic (D – harbour sediment), combustions products of domestic origin (charcoal, wood burning in fireplaces, etc.), forest fires (E, F and H) and are consistent with the mixed petrogenic and pyrolytic PAHs sources mentioned before. Although these were not similar to the signatures explaining the variance in the sediments in the same period (see section 2.4), the influence of boat traffic is recognised. The increase in recreational and fishing boat traffic in the Ria Formosa in the summer was already mentioned (section 2.4). Due to tourism, there is also a large increase (almost 2-fold) in the population around the Ria Formosa Formosa in the summer and a large increase in recreational and fishing boat traffic in the Ria Formosa in this season (Instituto da Água, 1994; ANA, 1995). These factors are probably responsible for the increase in PAH concentrations in the clam tissues.

In October, clams from sites 1 to 4 have a different PAHs distribution pattern (Figure 3.3 and 3.6). In clams from other sites, signatures K and P are the most relevant. Signature K (diesel soot) is also related to boat traffic. It is highest in clams from sites near the harbours of Olhão (site 6) and Tavira (site 7), respectively. The Olhão harbour moors around 200 fishing and 500 recreational boats in this time of year. The Tavira harbour is smaller, mooring 90 fishing and 450 recreational boats. Signature P, was found in Prince William Sound sediments exposed to the oil released after the Exxon Valdez spill (Alaska North Slope Oil), after suffering two years of natural weathering processes (Page *et al.*, 1999). It is clearly petrogenic in source and characteristic of oil contamination. This type of signature was found in almost all clam samples and with less weathering extent also in most of the sediment samples and has probably the same origin: hydrocarbons released inside the Ria Formosa or in the South Portuguese Coast (Vieites *et al.*, 2004; CCDR, 2005).

The same signatures of the August I data (D, E, F, H, P and S) were observed in November and December. The impact of the population on the Ria Formosa lagoon usually

decreases in this time of year and thus, PAH concentrations were lower (Figure 3.2). In clams from site 4 in November the signature was similar to fresh oil although signature L did not explain any of the variance in the samples from sites 2 and 3. It is possible that in these samples the PAH signature was modified.

In January, most of the variance in the data was explained by signatures related to weathered oil (O, P and R). These signatures were particularly important in clams from sites 1 and 2, located near the lagoon entrance and thus being affected by boats going in and out of the lagoon. At site 6, signature D (harbour sediment) in clam tissues was particularly representative. This is not strange, since site 6 is located near the Olhão harbour. Besides being used by recreational and fishing boats, this harbour has intense boat traffic and is also used by some commercial ships for the discharge of sand and fuel.

The variance in August II samples was explained to some extent by signatures E and F (pyrolytic), which is consistent with the PAH distribution pattern (Figure 3.3). Signature E is associated with forest fires which are common in this time of year (Burns *et al.*, 1997). For instance, in 1995 around 12000 ha of forest (composed mainly of cork and holm oak, eucalyptus and pine tree) burned in the Algarve region (CCDR, 2005). Signature F, however, is characteristic of organic matter combustions associated with domestic activities (Burns *et al.*, 1997) and may result from the impact of population increase due to tourism. Although not readily apparent from the PLS signature, pyrolytic PAHs were also detected in clam tissues in August II, while some variance is also explained by petrogenic sources (signatures L, O and P). Signatures O and P are respectively moderately and heavily weathered oil signatures found in sediment samples (Table 2.6). The appearance of these signatures points once more to an oil contamination of the Ria Formosa. The highest tPAH concentration in clam tissues was at site 8. At this site signatures C, J and K explain most of the variance in the data. Signature J is an unmodified oil signature while K is a diesel soot signature. These signatures

are probably related to boat traffic since they are generally associated with PAHs released from engines (Burns *et al.*, 1997).

The clam PAH distribution was compared with the sediment PAHs content (Chapter 2) and biota-sediment accumulation factors (BSAF) were calculated. In *R. decussatus*, BSAF values (Table A5 in Annexe) were much higher than those reported in the literature (see Table 1.5 for comparison) for the same PAHs in the mussel *Mytilus edulis* (Baumard *et al.*, 1999a) or the *Mercenaria mercenaria* (Nasci *et al.*, 1999) indicating that the sediments were less representative of the environmental PAH load than the clams. The lack of relationship between tPAH and individual PAH concentrations between clam tissues and the sediments (Figs. 3.9 and 3.12), especially in August, suggests that in this period the sediments were not the main PAHs source to clams. Instead, the water and/or food may be the most important pathways. Uptake of organic contaminants, like the PAHs, may proceed via several ways. These include absorption by direct partitioning of the dissolved contaminants through the gills or ingestion of small size particles (either food or sediment particles) and absorption through the digestive system. It is believed that the main route of uptake for the more water soluble PAHs is through water while the more hydrophobic PAHs are taken up mainly through ingestion of food or sediment (Meador *et al.*, 1995; Narbonne *et al.*, 1999). Water was considered the most important pathway for trybutyltin accumulation in this clam species: *R. decussatus* accumulated more than 90% of tributyltin (TBT) from the dissolved phase than from the sediments (Coelho *et al.*, 2002a). However, it was demonstrated that a small percentage of TBT bound to algal cells was also accumulated (Coelho *et al.*, 2002b). As TBT, PAHs are hydrophobic and their route of uptake by clams is not yet fully understood.

The difference in behaviour of the August clams (Figure 3.6) was also confirmed by the significant higher slope between log BSAF and K_{ow} (Figure 3.10), than in the other months. That is, clams accumulated the less soluble PAHs more than the more soluble ones or

eliminated these more rapidly. The accumulation of PAHs in bivalve tissues is also the result of an equilibrium between the hydrocarbons uptake and elimination, both being generally inversely related to the compounds K_{ow} (Meador *et al.*, 1995; Sericano *et al.*, 1996; Neff, 2002). That is, the more soluble PAHs (with lower K_{ow}) are more readily absorbed and easily eliminated by the organisms than the less soluble ones. The more hydrophobic PAHs need to be metabolised first in order to be eliminated from the organism (Neff, 2002). For instance, the freshwater clam *Corbicula fluminea* exposed to sediment particles contaminated with different PAHs, presented higher uptake and elimination rates for the more soluble PAHs (3 aromatic rings) than for the less soluble 4+ aromatic ring PAHs. Two days after exposure, only the less soluble PAHs (5 aromatic rings) remained in the tissues. For these PAHs, elimination began only 15 days after the exposure (Narbonne *et al.*, 1999). Thus, both the uptake and elimination processes favour the accumulation of the high molecular weight (less soluble) PAHs in bivalve tissues. Indeed, most of the clams collected from the Ria Formosa during this period have higher proportions of the 4+5+6 aromatic ring PAHs in relation to the 2+3 ring PAHs (Figure 3.3). Also, the arrangement of the individual PAHs expressed as proportions of tPAH in the PCA was by aromatic ring numbers, translating also the difference in solubility and thus modes of uptake and elimination of these compounds by clams (Figure 3.5). Most of the samples were placed in the upper quadrants of the scores PCA (Figure 3.5) reflecting the accumulation of higher proportions of the 4 aromatic ring PAHs. The only exceptions were the clams collected in August II, when a higher accumulation of the 5+6 aromatic ring PAHs occurred, and in autumn (October and November) when clam tissues were enriched in the 2+3 ring PAHs (Figure 3.3 and 3.6).

PAHs are even less soluble than TBT (Inaba *et al.*, 1995; Langston & Pope, 1995) and are known to adsorb to suspended particles including plankton (Kowalewska & Konat, 1997; Kowalewska, 1999; Okay *et al.*, 2000; Kirso *et al.*, 2001). PAHs sorbed to phytoplankton

cells are transferred with the detritus formed after cells death to the sediments where, depending on the environmental conditions, PAHs may either be buried (sedimentation) or released becoming available to filter-feeding or suspension-feeding organisms in natural aquatic ecosystems (Broman *et al.*, 1990). These contaminated particles could serve as a possible route for the more hydrophobic PAHs uptake (Narbonne *et al.*, 1999; Okay *et al.*, 2000). In fact, in mussels *M. edulis* simultaneously exposed to BaP (2 and 50 μg l^{-1}) and algae *Isochrysis galbana* (30 000 and 150 000 algal cells ml^{-1}), BaP accumulation was shown to be dependent on both BaP and algal concentrations, with the 5-fold increase in algal concentration being responsible for a 2-fold increase in the BaP accumulation in the mussel tissues (Okay *et al.*, 2000).

The consumption of PAH contaminated clams raises some concern for human health. In Portugal, legislation does not exist regarding PAH concentrations in clam or other shellfish tissues. However, some PAHs (naphthalene, anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*ghi*]perylene and indene[123-*cd*]pyrene) were recognized as priority dangerous substances by the Portuguese legislation and included in a list of Priority Substances (Annexe X) of the Water Framework Directive, to be analysed in water, sediments and biota (EU Directive 2000/60/CE). However, no limits were established. More recently, naphthalene and anthracene concentrations were restricted to 1.5 mg l^{-1} in sewage effluents (Portaria nº 50/2005). Therefore, the guidelines developed by the Oregon Health Division (USA) were used in this work for *R. decussatus* as mentioned in the results section (Gilroy, 2000). These guidelines limit the amount of benzo[*a*]pyrene (BaP) equivalents (in $ng.g^{-1}$ ww) in shellfish edible tissues as follows:

i) if the shellfish tissue contains less than 10 $ng.g^{-1}$ ww BaP eq, shellfish may be considered safe and acceptable for human consumption;

ii) if any shellfish tissue contains more than 45 $ng.g^{-1}$ ww BaP eq, shellfish will be considered unsafe for human consumption;

iii) if the tissue contains more than 10 $ng.g^{-1}$ but less than 45 $ng.g^{-1}$ ww BaP eq, there is a need for further monitoring of these samples.

Regarding PAH body burden in *R. decussatus*, they were in general safe for consumption. However, there was an exception in both August I and August II, when levels exceeded those considered safe for human consumption and corresponded to samples with high predominance of 5+6 aromatic hydrocarbons, which are the most carcinogenic PAHs (Gilroy, 2000). However, it is strongly recommended that a regular control of the PAHs content in clam tissues be implemented.

In summary, similarly to what was found for the sediments (Chapter 2), PAHs in clam whole soft tissues vary seasonally rather than spatially, being also associated with changes in PAH sources. Boat traffic, especially in the summer, appears to be the most relevant PAH source to the Ria Formosa. The seasonal variation of PAH concentrations in clam tissues, however, was distinct from that of the sediments and there was a lack of relationship between tPAH concentrations in both compartments. As a result, clams may better reflect the PAH load in the Ria Formosa than the sediments. Clams were generally considered safe for human consumption through out most of the year, with the exception of the summer suggesting the need for a regular survey of PAHs in clam tissues.

4. OXIDATIVE STRESS IN THE DIGESTIVE GLAND OF THE CLAM *Ruditapes decussatus*

4.1. INTRODUCTION

The use of organisms as bioindicators provides an indication of the bioavailable fraction of contaminants in the environment (Baumard *et al.*, 1998a). Suspension-feeder bivalves that accumulate and concentrate in their tissues hydrophobic contaminants (such as the polycyclic aromatic hydrocarbons), are often used as sentinel organisms to monitor the presence of these compounds in marine and estuarine systems (Goldberg & Bertine, 2000; Solé *et al.*, 1994; Porte *et al.*, 2000; Serafim & Bebianno, 2001). However, the concentration of contaminants in tissues alone does not give information on the impact of such compounds in the aquatic life. Therefore, several authors highlighted the importance of using an integrated approach to assess environmental quality and effects of toxicants on organisms (Livingstone *et al.*, 1995; den Besten, 1998; Nasci *et al.*, 2000). The use of biomarkers was thus introduced (Moore *et al.*, 2004).

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants, with a significant environmental impact since they are carcinogenic (Ohnishi & Kawanishi, 2002; Schneider *et al.*, 2002) and toxic to several marine species (Nasci *et al.*, 2000; French, 1998). The origins and sources of these compounds were described in Chapter 1. Following exposure to these contaminants, organisms tend to metabolise and eliminate them as metabolites, minimizing cellular damage. Such protective mechanisms involve the Cytochrome P450 system. Often, associated with this system are redox cycling mechanisms that generate oxyradicals (Livingstone *et al.*, 1990; Canova *et al.*, 1998). The extent to which oxyradicals produce biological damage is dependent on the effectiveness of antioxidant defence systems. These protective mechanisms involve a number of antioxidant enzymes, namely, superoxide dismutase (SOD; EC. 1.15.1.1), catalase (CAT; EC. 1.11.1.6) and

glutathione peroxidases (GPx; EC. 1.11.1.9), total fraction and selenium dependent (Livingstone *et al.*, 1992; Doyotte *et al.*, 1997), which have been proposed as biomarkers in a variety of marine organisms (Livingstone *et al.*, 1992; Gamble *et al.*, 1995; Regoli *et al.*, 1998). When antioxidant defences are unable to cope with the generation of oxyradicals there is an imbalance between the production and removal of oxidants, a situation known as oxidative stress (Livingstone *et al.*, 1990; Canova *et al.*, 1998). Oxidative stress includes oxidative damage, enzyme inactivation, protein degradation, DNA damage and lipid peroxidation (Halliwell & Gutteridge, 1986). In particular, lipid peroxidation (LPO) is considered a major mechanism, by which oxyradicals can cause tissue damage, leading to impaired cellular function and alteration in physicochemical properties of cell membranes, which in turn disrupt vital functions (Barata *et al.*, 2005).

Antioxidant enzyme activities determined in several bivalve species are related to exposure to inorganic and organic xenobiotics (Solé *et al.*, 1994). Although their role as biomarkers of PAHs mediated oxidative stress has been investigated (see section 1.2 and table 1.8), there are still some reservations on their application in field studies (Nasci *et al.*, 2000; Lionetto *et al.*, 2003; De Luca-Abbott *et al.*, 2005).

The clam *Ruditapes decussatus* (Linnaeus, 1758) is a suspension feeder sediment-dwelling bivalve, widely distributed through the Atlantic coast from England to Africa, in the south of the Mediterranean and West Africa (Roméo & Gnassia-Barelli, 1995; Bebianno *et al.*, 2004). Due to its feeding behaviour and low metabolic rate (Porte *et al.*, 2000; Solé *et al.*, 2000), *R. decussatus* accumulates in its tissues a large number of inorganic (metals) and organic (tributyltin, polychlorinated biphenyls and PAHs) compounds reflecting the bioavailable fraction of contaminants in the environment (Bebianno *et al.*, 2004). For this reason, this species has been used as a bioindicator species of inorganic (Moraga *et al.*, 2002; Bebianno & Serafim, 2003; Beiras & Albentosa, 2004; Smaoui-Damak *et al.*, 2004) and

organic contaminants (Solé *et al.*, 1994; Porte *et al.*, 1996; Ferreira & Vale, 1998; Porte *et al.*, 1998; Solé, 2000b; Solé *et al.*, 2000; Dellali *et al.*, 2001; Coelho *et al.*, 2002c; Binelli & Provini, 2003). Therefore, the activities of antioxidant enzymes (SOD, CAT, T GPx and Se GPx) as well as lipid peroxidation products were studied in *R. decussatus* digestive gland, the main tissue responsible for the biotransformation of lipophilic xenobiotics. The oxidative stress-related parameters with the PAHs content in clam whole soft tissues assessed the use of these enzymes as a measure of biological effect of PAHs.

Studies on biomarkers indicated the need to integrate interactions of abiotic (temperature, salinity, turbidity, diet, etc.) and biotic factors (reproduction cycle, growth, age, sex, etc.) (Viarengo *et al.*, 1991; Solé *et al.*, 1995; Vidal *et al.*, 2002b; Bodin *et al.*, 2004). Evaluation of the variation of biomarkers in model organisms collected in specific habitats is thus recommended in order to distinguish between pollution-related effects and natural fluctuations (Cajaraville *et al.*, 2000).

Therefore, clams were collected in the Ria Formosa lagoon over one year period from the same sites described in the previous chapters, to integrate the variations of environmental parameters and evaluate the effects of PAHs.

4.2. MATERIALS AND METHODS

4.2.1. Sampling

Clams were sampled monthly, at low tide, for one year, from August I (August 94) to August II (August 95), in the sites mentioned in section 2.1.1 (Figure 2.1). Samples were collected at the eight sites in August I, October, November, December, January, May and August II and only at four sites (sites 1, 4, 6 and 7) in March, June and July, due to logistic reasons.

The organisms were kept alive at 4ºC prior to the arrival at the laboratory (less than 2 hours). Upon arrival at the laboratory, they were kept in aerated seawater for 2 days to clear gut content (Livingstone *et al.*, 1989). Previous studies on antioxidant enzyme variability on the same species indicated that in the first two days these enzymes were not significantly affected (Geret & Bebianno, 2004).

4.2.2. Tissues preparation

Clams were dissected and digestive glands excised. Imediately after dissection tissues were homogenised in pools of five digestive glands in a 20 mM Tris-HCl buffer (pH 7.6) containing 1 mM EDTA, 0.5 M sucrose, 0.15 M KCl and 1 mM dithiothreitol, using an Ultra-Turrax T 25 homogeniser. Homogenates were centrifuged at 500 g for 15 minutes to remove remaining tissues and the resulting supernatant was centrifuged at 12,000 g, for 45 min, to separate the mitochondrial and cytosolic fractions. The mitochondrial fractions were resuspended in the homogenisation buffer, re-centrifuged for washing and kept at 4ºC overnight without resupension. Immediately before analysis they were resuspended in 1 ml of homogenisation buffer. The cytosolic fractions were passed down through a Sephadex G25 column (PD10 Pharmacia) to remove interfering small weight proteins and kept at –80ºC until analysis.

Total protein and LPO levels were analysed in the digestive gland of a different set of organisms due to the interference of sucrose on protein assay. After dissection, tissues of four clams were homogenised separately in 20 mM Tris-HCl buffer (pH 8.6) containing 150 mM NaCl and centrifuged at 3000 g for 15 min. The analyses were carried out in the supernatant.

All the procedures were performed at 4ºC.

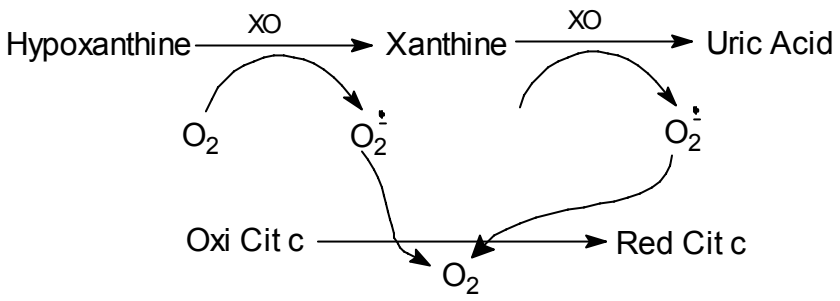
All reagents were purchased from SIGMA with the exception of the phosphates that were purchased from Merck.

4.2.3. Biochemical Analysis

All enzyme activities were measured in triplicate at 20ºC using a double beam spectrophotometer (Hitachi U – 2000).

A. Superoxide Dismutase

SOD activity was measured according to McCord & Fridovich, 1969) in both the mitochondrial and cytosolic fractions. It is expressed as units (U), where 1 U is the amount of sample required to cause 50% inhibition of the rate of reduction of cytochrome *c* by the superoxide anion generated by the xanthine/hypoxanthine system:



The reaction mixture contained 43 mM KH_2PO_4/K_2HPO_4 (pH 7.8), 10 µM cytochrome *c*, 0.1 mM EDTA, 50 µM hypoxanthine, 1.8 mU/ml xanthine oxidase and 100 µl of cytosolic or mitochondrial fraction of the digestive gland homogenate, in a final volume of 3 ml. When less amount of sample was required to cause 50% of inhibition the 100 µl volume was complemented with phosphate buffer. The reduction of the cytochrome *c* was measured at 550 nm and the reaction started by the addition of xanthine oxidase. The activity of SOD is expressed in U mg^{-1} of total protein.

B. Catalase

Catalase activity was measured also in both mitochondrial and cytosolic fractions and the activities summed. The decrease of hydrogen peroxide (H_2O_2) absorbance at 240 nm was measured (Greenwald, 1985). The reaction mixture contained 50 mM KH_2PO_4/K_2HPO_4 (pH 7.5), 50 mM H_2O_2 and 100 µl of mitochondrial or cytosolic fraction in a final volume of 3 ml. The reaction started by the sample addition. Catalase activity is expressed in µmol min^{-1} mg^{-1} of total protein, using a molar extinction coefficient of 40 $M^{-1}cm^{-1}$.

C. Glutathione Peroxidases

Total GPx and Se-dependent GPx activities were measured only in the cytosolic fractions and were determined as described by Lawrence & Burk, 1976) according to the scheme below, where the reduction of GSSG was linked to the oxidation of NADPH at 340 nm using added GR.

$$2\ GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$$

$$GSSG + 2\ NADPH \xrightarrow{GR} 2\ GSH + 2\ NADP^+$$

Cumene hydroperoxide and H_2O_2 were used as substrates (ROOH) for T GPx and Se GPx, respectively. For the determination of T GPx the enzymatic assay was composed of 65 mM KH_2PO_4/K_2HPO_4 (pH 7.5), 2 mM GSH, 1 U GR, 0.12 mM NADPH, 4 mM cumene hydroperoxide and 100 µl of cytosolic fraction in a 1 ml final volume. For Se GPx the enzymatic assay contained 65 mM KH_2PO_4/K_2HPO_4 (pH 7.5), 2 mM GSH, 1 mM sodium azide, 1 U GR, 0.12 mM NADPH, 2 mM H_2O_2 and 100 µl of cytosolic fraction in a final volume of 1 ml. The reaction was started by the addition of the hydroperoxide, in both assays. GPx activities are expressed in nmol min^{-1} mg^{-1} of total protein using a molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$.

D. Total protein

Total protein concentrations were determined by the method of Lowry (Lowry *et al.*, 1951). Briefly, 2.5 ml of a 0.1 N NAOH solution containing 2% Na_2CO_3, 0.01% $CuSO_4$ and 0.02% Na-K tartarate, and 0.25 ml of the Folin-Ciocalteau reagent were added to 0.5 ml of diluted homogenate (1:50). The absorbance of the resulting chromophore was measured at 750 nm and quantification performed with a calibration curve prepared with bovine serum albumin (BSA) solution. Protein concentrations are expressed as mg g^{-1} wet weight.

E. Lipid Peroxidation

Lipid peroxidation was determined in the same homogenates as the total protein. The method, described by Erdelmeier *et al.* (1998), measures the amount of malondialdehyde (MDA) and 4-hydroxyalkenals (4,HNE) produced in the peroxidation of membrane lipids. Briefly, 200 µl of the 3,000 g centrifugation supernatant were mixed with 650 µl of a mixture of 6 ml of methanol with 18 ml of 10.3 mM N-methyl-2-phenylindole and with 150 µl of 15.4 M methanesulfonic acid. The mixture was incubated at 45ºC for 60 min, and then centrifuged at 15,000 g for 10 min. The absorbance of the reaction mixture was measured at 586 nm against a blank containing buffer and reagents. Lipid peroxidation is expressed as nmol MDA + 4-HNE g^{-1} of total protein.

4.2.4. Statistical Analysis

Data are presented as mean ± one standard deviation. Parametric one-way analysis of variance tests with *post-hoc* comparisons were used to test differences between groups in parametric sets of data. When possible, non-parametric data were log transformed in order to obtain normal distribution. In non-parametric sets of data, the non-parametric Kruskall-Wallis

and Mann-Whitney U-tests were applied. Computer program STATISTICA for Windows Release 5.1 (StatSoft, Inc., Tula, OK) was used.

The relationship between enzyme activities and PAH concentrations in clam tissues was analysed by Spearman Ranks Correlation (for non-parametric data) and PLS analysis. Not detected data were substituted by half the limit of detection. The Spearman Correlation gives an indication of the enzymes response in relation to PAH concentrations in clam tissues. In PLS analysis, the clam PAH concentrations or proportion data were used as an X-block and the stress related markers as the Y-block. PLS analysis results in model coefficients for the variables, called PLS-weights. The weights for the X-variables, denoted *w*, indicate the importance of these variables, how much they "in a relative sense" participate in the modelling of Y. The weights for the Y-variables, denoted by *c*, indicate which Y-variables are modelled in the respective PLS model dimensions. When these coefficients are plotted in a so-called "*wc*" plot, a picture showing the relationships between X and Y is obtained.

Significant differences and correlations were considered when $p<0.05$.

4.3. RESULTS

4.3.1. Antioxidant Enzymes

A. Superoxide Dismutase

SOD activity measured in both the mitochondrial (Mit SOD) and cytosolic (Cyt SOD) fractions of the clam digestive gland are shown in Figure 4.1 (see also Table A6 in Annexe). Mean SOD activities ranged from 1.01±0.00 to 33.4±2.5 U mg^{-1} protein in the mitochondrial fraction and from 2.67±1.53 to 101±7 U mg^{-1} protein in the cytosolic fraction (Table A6 in Annexe).

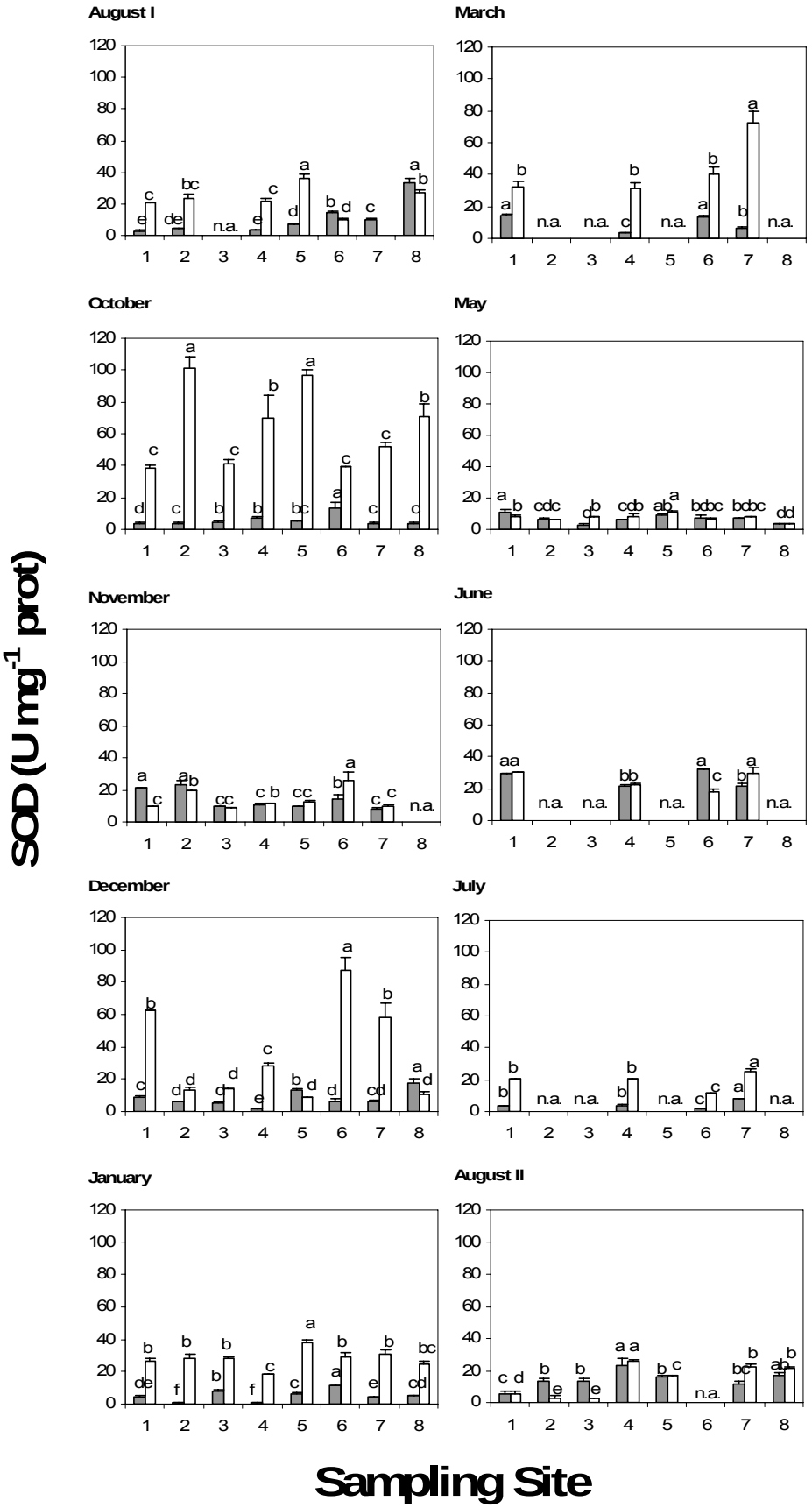


Figure 4.1 – Spatial and seasonal variation of mitochondrial (grey) and cytosolic (open) SOD activity (mean±standard deviation) in the digestive gland of *Ruditapes decussatus* (bars labelled with the same letter are not statistically different). n.a. – data not available.

The median of Mit and Cyt SOD activities was calculated monthly in order to provide a threshold value for these enzymes in the digestive gland of the clams from the different sites. The median was chosen, because data was not normally distributed.

Mit SOD was significantly lower than Cyt SOD in October, December, January, March and July ($p<0.05$). However, in August I, November, May, June and August II, activities were similar ($p>0.05$).

Spatial differences in SOD activities were observed monthly in both fractions ($p<0.05$). However, considering the whole data set, no significant differences occurred in Mit or Cyt SOD in the clams digestive glands between sites ($p>0.05$). Seasonal variations of Mit and Cyt SOD activities were significantly different, that is, these enzymes behaved differently throughout the year.

Seasonally, Mit SOD activity in *R. decussatus* digestive gland was greatest in June ($p<0.05$). In August I, the activity ranged from 2.90±0.41 to 33.4±2.5 U mg^{-1} prot. In this month, Mit SOD activity was significantly induced in clams from sites 6 to 8 while clams from sites 1, 2 and 4 had the lowest activities ($p<0.05$).

In October, Mit SOD activity significantly decreased when compared to August I (3.54±0.83 – 13.8±2.9 U mg^{-1} prot) and was induced in the digestive gland of clams from sites 4 to 6. The lowest activities were in the digestive gland of clams from site 1 ($p<0.05$). In November, Mit SOD activity increased again reaching significant higher activities than in the previous month (8.39±0.62 – 23.2±2.3 U mg^{-1} prot). Contrary to what occurred in October, Mit SOD activity was induced in the digestive gland of *R. decussatus* from sites 1 and 2 ($p<0.05$). Mit SOD activities decreased again in December (1.58±0.57 – 18.0±2.3 U mg^{-1} prot) reaching values similar to those in October ($p>0.05$). Mit SOD was again induced in clams from site 1 and also from sites 5 and 8 while the lowest activity was in the digestive gland of clams from site 4 ($p<0.05$). Afterwards, from January until May, Mit SOD activities

in the digestive gland of *R. decussatus* remained unchanged varying between 1.01±0.00 and 14.0±1.2 U mg^{-1} prot with levels similar to those of December but, during this period, the spatial variation had a different pattern each month. In January, Mit SOD activity increased in the digestive gland of clams from sites 3, 5 and 6, while in the digestive gland of clams from sites 2 and 4 was lowest ($p<0.05$); and in May, Mit SOD activity increased in the digestive gland of clams from sites 1 and 5 ($p<0.05$).

The greatest Mit SOD activities (21.3±1.0 – 32.2±0.0 U mg^{-1} prot) ($p<0.05$) were in June, but, in July it significantly decreased (1.95±0.23 – 8.08±0.29 U mg^{-1} prot), increasing again in August II (5.58±1.62 – 22.9±5.1 U mg^{-1} prot), when levels were similar to those in November. In this month, Mit SOD was induced in the digestive gland of clams from site 4, 5 and 8 while the lowest activities were in clams from sites 1 and 7 ($p<0.05$).

In general, there was no relationship between the spatial distribution of Mit SOD activity in the digestive gland of *R. decussatus* and that of tPAH concentrations in the clam whole soft tissues (Chapter 3), with an exception in October, when both Mit SOD activity and tPAH concentrations were highest in clams from site 6 and lowest in those from site 1 ($p<0.05$).

Contrarily to Mit SOD, Cyt SOD activity in the clam digestive glands, was significantly higher in October and lower in November, May, July and August II ($p<0.05$) (Figure 4.1; Table A6). In August I, the activity ranged from 10.3±0.5 to 36.5±2.7 U mg^{-1} prot and was induced in clams from sites 5 and 8, while the lowest activity was in clams from site 6 ($p<0.05$). In October Cyt SOD activity significantly increased (38.9±1.7 – 101±7 U mg^{-1} prot) reaching its greatest levels. Cyt SOD was again increased in clams from site 5 along with those from site 2 and the lowest Cyt SOD activity remained on clams from site 6 and in those from sites 1 and 3 ($p<0.05$). Cyt SOD activity reached its lowest levels in November (8.49±0.45 – 25.6±5.1 U mg^{-1} prot). Contrary to the spatial distribution of the previous

months, Cyt SOD activity was now higher in clams from sites 2 and 6 although the lowest activities remained in those from sites 1 and 3, along with those from sites 5 and 7 ($p<0.05$). In December, Cyt SOD activity in the digestive gland of *R. decussatus* significantly increased once more (9.03±0.00 – 87.3±7.9 U mg^{-1} prot) remaining unchanged in January (18.7±0.0 – 37.8±2.3 U mg^{-1} prot) and March (31.1±4.2 – 72.7±7.0 U mg^{-1} prot). Cyt SOD activity was induced in clams from sites 1, 4, 6 and 7 in December; and in those from sites 5, 6 and 7 in January and the lowest activities were in clams from sites 2, 3, 5 and 8 in December and 4 in January ($p<0.05$). In May, Cyt SOD activity decreased again (3.39±0.00 – 10.5±1.2 U mg^{-1} prot) and remained unchanged until August II (2.67±1.53 – 30.1±0.0 U mg^{-1} prot). The activity of this enzyme increased in clams from site 5 and was lowest in clams from site 8 ($p<0.05$). In August II though, Cyt SOD activity was greatest in clams from site 4 and lowest in clams from sites 2 and 3 ($p<0.05$).

Similarly to Mit SOD, no relationship exists between Cyt SOD activity in the clam digestive glands and tPAH concentrations in clam whole tissues (Chapter 3).

B. Catalase

Spatial and seasonal variation in CAT activity is in Figure 4.2. Mean CAT activities ranged from 32.8±1.1 to 617±34 μmol min^{-1} mg^{-1} protein (Table A7 in Annexe).

Spatially, significant differences exist between CAT activities in the digestive gland of clams from site 6 (111±2 – 617±34 μmol min^{-1} mg^{-1} prot) and from sites 1, 3, 4, 5 and 8 (32.8±1.1 – 533±18 μmol min^{-1} mg^{-1} prot) ($p<0.05$). Seasonally, CAT activity was highest in the digestive gland of clams collected in December, March, June and July and lowest in those collected in August (I and II) and in January ($p<0.05$).

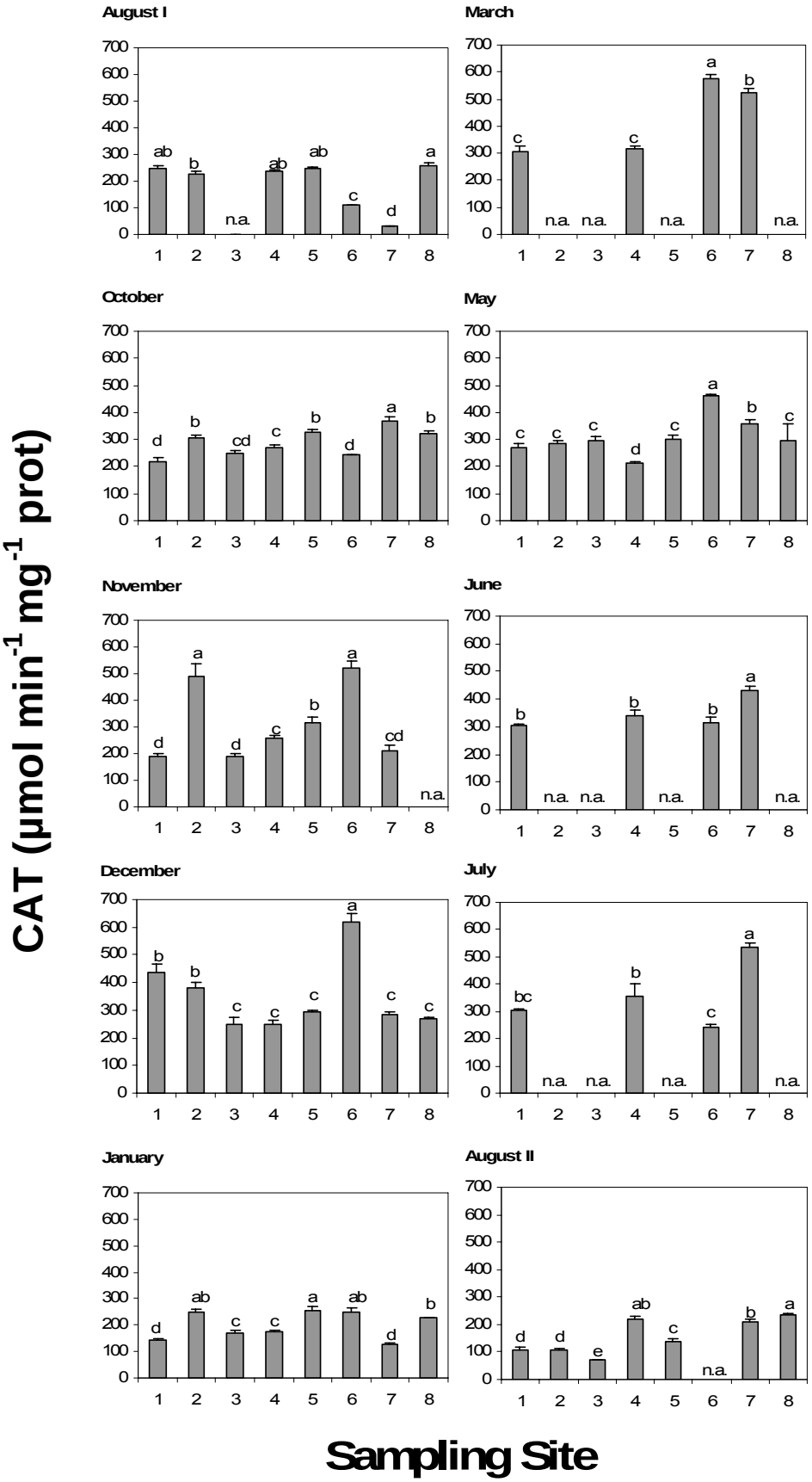


Figure 4.2 – Spatial and seasonal variation of CAT activity (mean±standard deviation) in the digestive gland of *Ruditapes decussatus* (bars labelled with the same letter are not statistically different). n.a. – data not available.

In August I, CAT activities were relatively low (32.8±1.1 – 257±13 μmol min^{-1} mg^{-1} prot) but significantly increased in October (219±14 – 368±13 μmol min^{-1} mg^{-1} prot) ($p<0.05$). In August, the activity was lowest in clams from site 7 ($p<0.05$). In October, however, CAT was induced in clams from sites 2, 5, 7 and 8 while the lowest CAT activities were in clams from sites 1 and 6 ($p<0.05$). In November (189±11 – 523±21 μmol min^{-1} mg^{-1} prot) and December (247±18 – 617±34 μmol min^{-1} mg^{-1} prot) CAT activity in the clams digestive gland was similar to that of October ($p>0.05$). The spatial distribution, however, was slightly different in these months; in November, CAT was induced in the digestive gland of clams from sites 2, 5 and 6, while the lowest remained in clams from site 1 along with those from site 3; in December, CAT continued to be induced in clams from sites 2 and 6 along with those from site 1 and the lowest activity remained in clams from site 3, along with those from site 8 ($p<0.05$). In January, CAT activity significantly decreased (130±3 – 255±17 μmol min^{-1} mg^{-1} prot) reaching levels similar to those of August I. In this month, CAT was induced in clams from site 2, 5, 6 and 8, and the activity was lowest in clams from sites 1 and 7 ($p<0.05$). March presented the highest CAT activity (305±20 – 577±15 μmol min^{-1} mg^{-1} prot), though it significantly decreased in May (213±7 – 460±7 μmol min^{-1} mg^{-1} prot) ($p<0.05$). The spatial distribution in this month was, similar to that of March, that is, CAT activity was induced in clams from sites 6 and 7 and was the lowest in clams from site 4. CAT activity in the cla digestive glands remained unchanged until July (240±13 – 533±18 μmol min^{-1} mg^{-1} prot), decreasing only in August II (70.2±2.8 – 233±8 μmol min^{-1} mg^{-1} prot) to its minimum levels ($p<0.05$). CAT activity was now higher in the digestive gland of clams from sites 4, 7 and 8 and lower in clams from site 3 ($p<0.05$).

As observed for SOD, no relationship exists between the spatial distribution of CAT activity in the clam digestive glands and tPAH content of clam whole soft tissues (Chapter 3).

C. Total Glutathione Peroxidase

Figure 4.3 presents the spatial and seasonal variation in T GPx activities in the digestive gland of *R. decussatus*. Mean T GPx activities ranged between not detected (n.d.) and 55.2±1.9 nmol min^{-1} mg^{-1} protein (Table A8 in Annexe).

Spatially, T GPx activities were frequently higher in *R. decussatus* digestive gland from site 5; however, no significant differences exist between sites when the whole data set was considered ($p>0.05$). Still, a significant monthly spatial variation was observed. Seasonally, the greatest T GPx activities were in clams collected in November, June and July and the lowest in January ($p<0.05$).

In August I, T GPx activities were generally low, ranging from n.d. to 16.6±2.2 nmol min^{-1} mg^{-1} prot. T GPx activity was higher in clams from sites 1, 5 and 8, while the lowest activity was in clams from site 7 ($p<0.05$). In October, T GPx activity in the clam digestive gland (3.65±2.53 – 19.2±2.6 nmol min^{-1} mg^{-1} prot) was similar to that of August I ($p>0.05$) though it significantly increased in November (4.65±0.99 – 55.2±1.9 nmol min^{-1} mg^{-1} prot) when it reached its greatest activity ($p<0.05$). The spatial distribution of T GPx activity in the clam digestive glands was, however, distinct in these months. Similarly to August I, in October, T GPx activity increased in clams from sites 1, 5 and 7, while the lowest activities were in clams from sites 2, 6 and 8 ($p<0.05$). In October, T GPx activity in the clam digestive gland (3.65±2.53 – 19.2±2.6 nmol min^{-1} mg^{-1} prot) was similar to that of August I ($p>0.05$) though it significantly increased in November (4.65±0.99 – 55.2±1.9 nmol min^{-1} mg^{-1} prot) when it reached its greatest activity ($p<0.05$). The spatial distribution of T GPx activity in the clam digestive glands was, however, distinct in these months. Similarly to August I, in October, T GPx activity increased in clams from sites 1, 5 and 7, while the lowest activities were in clams from sites 2, 6 and 8 ($p<0.05$).

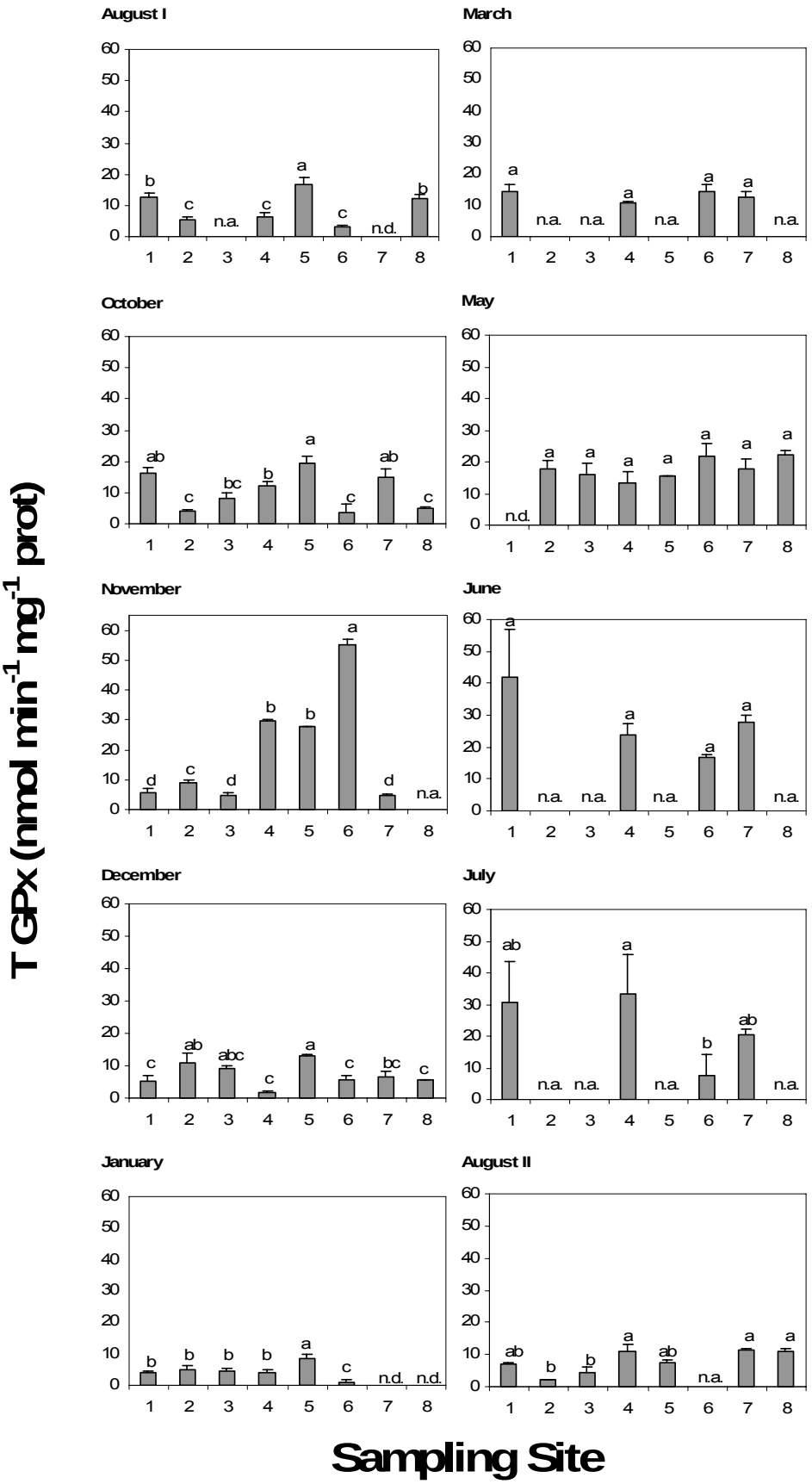


Figure 4.3 – Spatial and seasonal variation of T GPx activity (mean±standard deviation) in the digestive gland of *Ruditapes decussatus* (bars labelled with the same letter are not statistically different). n.d. – not detected; n.a. – data not available.

However, in November, T GPx was induced in clams from sites 4, 5 and 6 and the lowest activities were in clams from sites 1, 3 and 7 ($p<0.05$). After this month, T GPx activity in *R. decussatus* digestive gland significantly decreased, reaching a minimum in January (n.d. – 8.40±1.64 nmol min^{-1} mg^{-1} prot) ($p<0.05$). From January to May, T GPx activity increased once more being similar between sites ($p>0.05$). In June and July, T GPx activities in the clam digestive glands were similar (7.67±6.64 to 42.1±14.8 nmol min^{-1} mg^{-1} prot) ($p>0.05$) but, in August II, T GPx activity decreased again (2.06±0.27 – 11.3±0.4 nmol min^{-1} mg^{-1} prot) and was induced in clams from sites 4, 7 and 8 and lowest in clams from sites 2 and 3 ($p<0.05$).

Comparing the spatial distribution of T GPx activity in the clam digestive gland, within each month, with the equivalent spatial distribution in tPAH concentrations in the clam whole tissues, again no relationship exist.

D. Se dependent Glutathione Peroxidase

The spatial and seasonal variation of Se GPx activity in *R. decussatus* digestive gland is in Figure 4.4. Mean Se GPx activities were significantly lower than T GPx (n.d. to 45.0±12.9 nmol min^{-1} mg^{-1} protein) (Table A8 in Annexe).

Spatially, and similarly to Mit and Cyt SOD, no significant differences exist between sites when the whole data set was considered ($p>0.05$). However, monthly spatial variations were observed. Seasonally, like T GPx, Se GPx activity was significantly higher in June and July ($p<0.05$).

From August I to May, Se GPx activity was low ranging from n.d. to 8.28±0.56 nmol min^{-1} mg^{-1} protein.

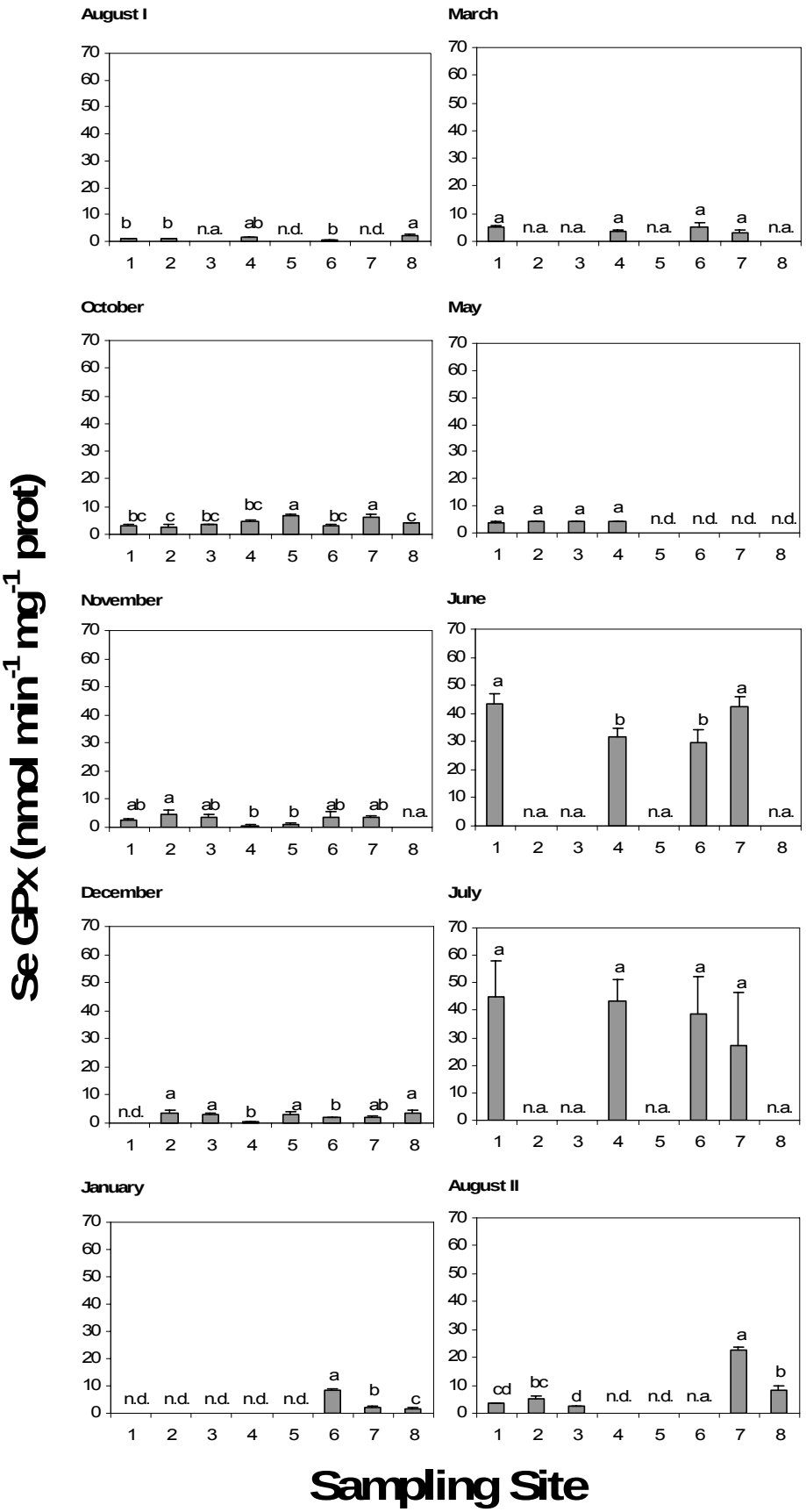


Figure 4.4 – Spatial and seasonal variation of Se GPx activity (mean±standard deviation) in the digestive gland of *Ruditapes decussatus* (bars labelled with the same letter are not statistically different). n.d. – not detected; n.a. – data not available.

The spatial variation of Se GPx activity, however, was different in each of these months: in August I, Se GPx activity was higher in clams from site 8 and similar in the other sites; in October, however, Se GPx was induced in clams from sites 5 and 7, while the lowest activities were in those from sites 2 and 8 ($p<0.05$); in November, Se GPx was now induced in clams from site 2 while the lowest activities were in clams from sites 4 and 5; in December, Se GPx remained high in clams from site 2, along with those from sites 3, 5 and 8 while the lowest activity was in clams from site 1 ($p<0.05$); in January, Se GPx activity in the clam digestive glands was only detected in clams from sites 6 to 8, being higher in clams from site 6 ($p<0.05$). In May, Se GPx was only detected in clams from sites 1 to 4 and no significant differences were observed among these sites ($p>0.05$). In June and July, Se GPx activity significantly increased (27.2±19.1 and 45.0±12.9 nmol min^{-1} mg^{-1} protein) compared to the previous months, reaching its highest levels ($p<0.05$). In August II, Se GPx activity significantly decreased again ranging from n.d. to 22.4±1.4 nmol min^{-1} mg^{-1} protein ($p<0.05$) and was highest in the digestive gland of clams from site 7 and lowest in clams from sites 4 and 5 ($p<0.05$).

When the spatial variation of Se GPx activity in the clam digestive glands, within each month, is compared to the corresponding spatial distribution of tPAH, no similarities are found.

E. Lipid Peroxidation

Mean levels (±S.D.) of lipid peroxidation (LPO) in the digestive gland of clams *Ruditapes decussatus* over one year are shown in Figure 4.5. No spatial variation exists. Seasonally, the highest LPO was in the digestive gland of clams collected in November and December while the lowest were in August (I and II), October and May ($p<0.05$). This

seasonal distribution appears to be the opposite of that of PAH concentrations in clam tissues (maximum in the summer).

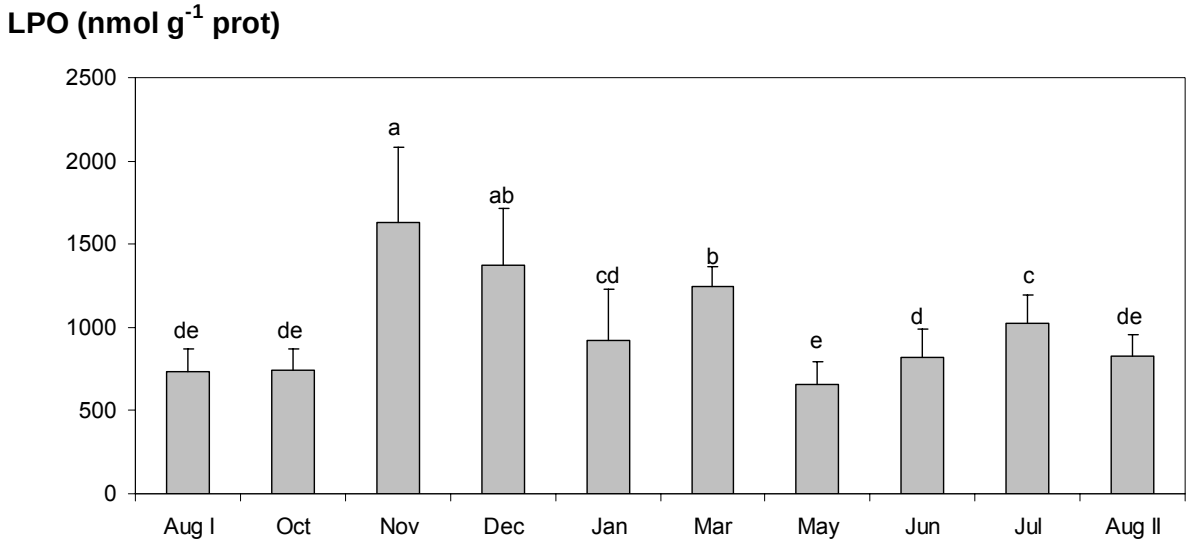


Figure 4.5 – LPO in the digestive gland of *Ruditapes decussatus* (bars labelled with the same letter are not statistically different).

4.3.2. Relationship between antioxidant enzymes and lipid peroxidation in *Ruditapes decussatus* digestive gland

The combined effect of the antioxidant action on all enzymes and LPO was evaluated by Spearman Rank Order Correlations (Table 4.1). No relationship exists between both SOD subcellular fractions (Mit and Cyt), confirming what was already anticipated by the spatial and seasonal variation analysis indicated above. However, a significant positive relationship exists between CAT and Cyt SOD activities ($p<0.05$). Both glutathione peroxidases were also positively correlated with each other (T GPx and Se GPx) and with CAT ($p<0.05$), indicating that the spatial and seasonal variation of these enzymes activity was similar ($p>0.05$), which highlights their mutual effort in antioxidant defence. LPO, however, was unrelated to any of the enzymes, meaning that the increase observed in oxidative damage in the winter was not related to induction or inhibition of these antioxidant enzymes.

Table 4.1 – Spearman Rank Order Correlation coefficients between antioxidant enzyme activities and MDA concentrations. Significant coefficients (p<0.05) are marked with *.

	Mit SOD	Cyt SOD	CAT	T GPx	Se GPx
Mit SOD					
Cyt SOD	-0.152				
CAT	0.034	0.308*			
T GPx	0.097	0.025	0.590*		
Se GPx	0.166	0.055	0.328*	0.342*	
LPO	-0.008	0.072	0.283	0.145	0.090

4.3.3. Relationship between Oxidative Stress and PAHs

The relationship between enzyme activity in the digestive gland of *R. decussatus* and PAH concentration in the whole soft tissues was analysed by Spearman Ranks Correlation (for non-parametric data) and PLS analysis.

In addition to the identification of significant relationships between these variables (activity of antioxidant enzymes and PAH concentrations), Spearman by Ranks Correlations were also used to identify the tendencies in antioxidant enzyme activities as a response to the variation in PAH concentrations. PLS analysis was also used to identify which PAHs signature (combination of individual PAHs as proportions of tPAH) was more associated with the variation of the antioxidant enzyme activity and lipid peroxidation.

In a first analysis, Spearman by Ranks correlations were performed monthly, thus analysing the antioxidant enzyme activities as a response to the spatial variation of PAH concentrations. However, almost all coefficients were not significant, meaning that the spatial distribution of the antioxidant enzyme activities, within each month, was not related to PAH concentrations. That was not the case, however, if the whole data set was used (Table A9 in Annexe).

Both Mit and Cyt SOD activities were unrelated to either individual or tPAH or even grouped by aromatic ring numbers. The relationships between CAT, T GPx, Se GPx, LPO

and the 4 and 5 aromatic ring PAHs were negative (Table A9), meaning that PAHs are inversely related to antioxidant enzyme activities and LPO.

CAT activity in the clam digestive gland was negatively correlated with tPAH in clam whole soft tissues (-0.560) as well as with most of the 3 ring PAHs: fluorene (-0.403), phenanthrene (-0.472) and anthracene (-0.393); all of the 4 ring PAHs: fluoranthene (-0.452), pyrene (-0.427), benzo[*a*]anthracene (-0.505) and chrysene (-0.330); and one of the 5 ring PAHs, benzo[*b*]fluoranthene (-0.273) ($p<0.05$).

Similarly, T GPx activity in *R. decussatus* digestive gland was negatively correlated with several individual PAHs in whole soft tissues namely the 3 aromatic rings and most of the 4 aromatic rings: phenanthrene (-0.288); anthracene (-0.304); fluoranthene (-0.390), pyrene (-0.353), benzo[*a*]anthracene (-0.415), chrysene (-0.385) and dibenzo[*a,h*]anthracene (-0.256) and indene[123-*cd*]pyrene (-0.278); and with tPAH concentrations (-0.417) ($p<0.05$).

Se GPx activity in the clam digestive gland was negatively correlated with the concentrations of two individual PAHs: phenanthrene (-0.269) and benzo[*a*]anthracene (-0.249) and with tPAH concentrations (-0.255) in clam whole soft tissues ($p<0.05$). However, contrary to the other antioxidant enzymes, positive relationships exist between Se GPx activity in clam digestive gland and the 5 and 6 aromatic ring PAHs, namely, benzo[*k*]fluoranthene (0.311), benzo[*a*]pyrene (0.321) and benzo[*g,h,i*]perylene (0.276) ($p<0.05$).

The relationships between LPO in the clam digestive gland and PAH concentrations in the clam whole soft tissues were negative: with fluorene (-0.419), phenanthrene (-0.517), anthracene (-0.408), fluoranthene (-0.383), pyrene (-0.465), benzo[*a*]anthracene (-0.481) and benzo[*b*]fluoranthene (-0.317). Additionally, LPO in *R. decussatus* digestive gland was negatively related to tPAHs (-0.444) concentrations in clam soft tissues ($p<0.05$).

Figure 4.6 shows the results of PLS analysis using the individual PAH concentrations in the whole soft tissues of clams as the X-variables and the stress related parameters in the digestive gland of, used as the Y-variables (the first component explained 43.2% and the second component 10.9% of the variance in the data, both significant). In this analysis, the X-variables (individual PAHs) that have the greatest loading factors have the greatest influence in the Y-variable (antioxidant enzymes).

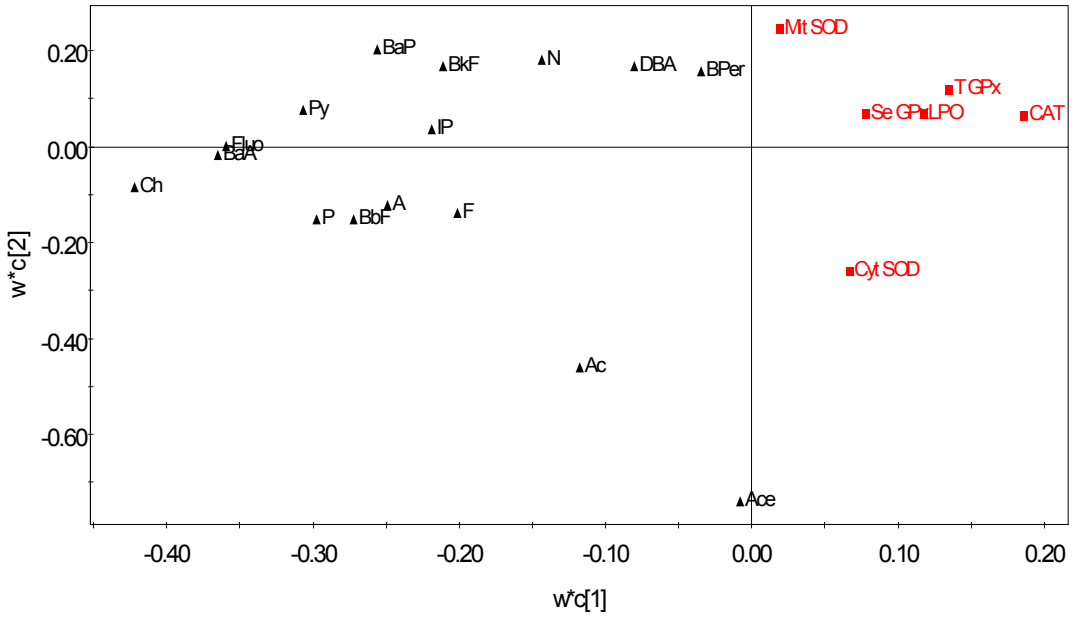


Figure 4.6 – PLS weights plot of the individual PAHs concentrations (X-variables) and the stress related parameters (Y-variables). The first component explained 43.2% and the second component 10.9% of the variance in the data (n = 65 *observations*).

As anticipated by the Spearman by Ranks Correlations, almost all PAH concentrations in clam whole soft tissues are inversely related with the antioxidant enzyme activities and LPO in the clam digestive gland, since they are in opposite sides of the plot. The 4 aromatic ring PAHs (Ch, Fluo, BaA and Py) appear to exert the greatest influence on all enzymes, especially CAT, which is the enzyme furthest apart from the origin.

Figure 4.7 shows the fraction of variance explained (grey bars) and predicted (open bars) by the PAH concentrations for each Y-variable. The individual PAH concentrations in

the clam whole soft tissues explain very small amounts of the variance within the antioxidant enzymes and LPO in the clam digestive gland. From all enzymes, CAT presents the highest explained variance. The amount of variance within the enzyme activities in the digestive gland of *R. decussatus* data predicted by the PAH concentrations in the whole soft tissues of clams was also low.

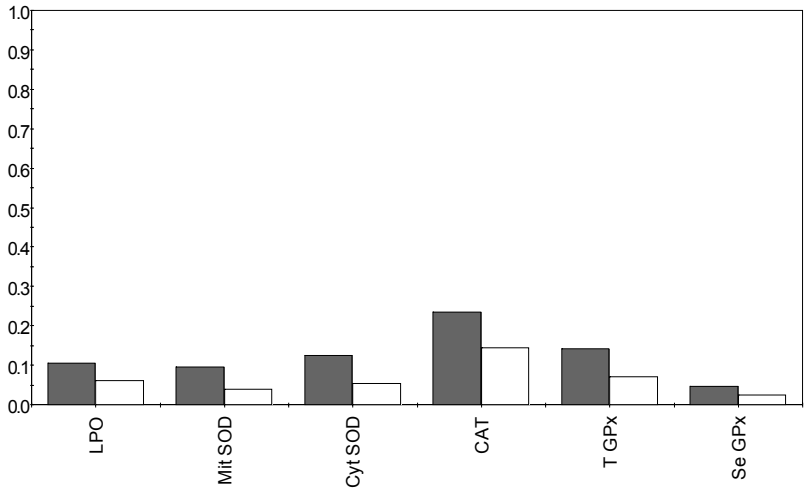


Figure 4.7 – Overview plot showing the individual cumulative R^2 (explained variation –grey bars) and Q^2 (predicted variation – open bars) for the Y-variables, using the individual PAHs concentrations as the X-variables.

When PAHs are expressed as proportions of tPAH (Figure 4.8), the obtained PLS results are considerably different. In this case, it is possible to see which PAHs are associated with the antioxidant enzyme activities. It appears that the enzymes are induced by PAH mixtures containing higher proportions of the low molecular weight hydrocarbons of 2 and 3 aromatic rings (Ace, Ac, F, A and P) and some of 4 rings (Fluo and Py) in the PAHs mixture or signature. The high molecular weight hydrocarbons, however, are located on the opposite side of the plot, which means that higher proportions of the 4 and 5 aromatic ring hydrocarbons in the PAHs mixture or signature are associated with a decrease in the antioxidant enzymes activity.

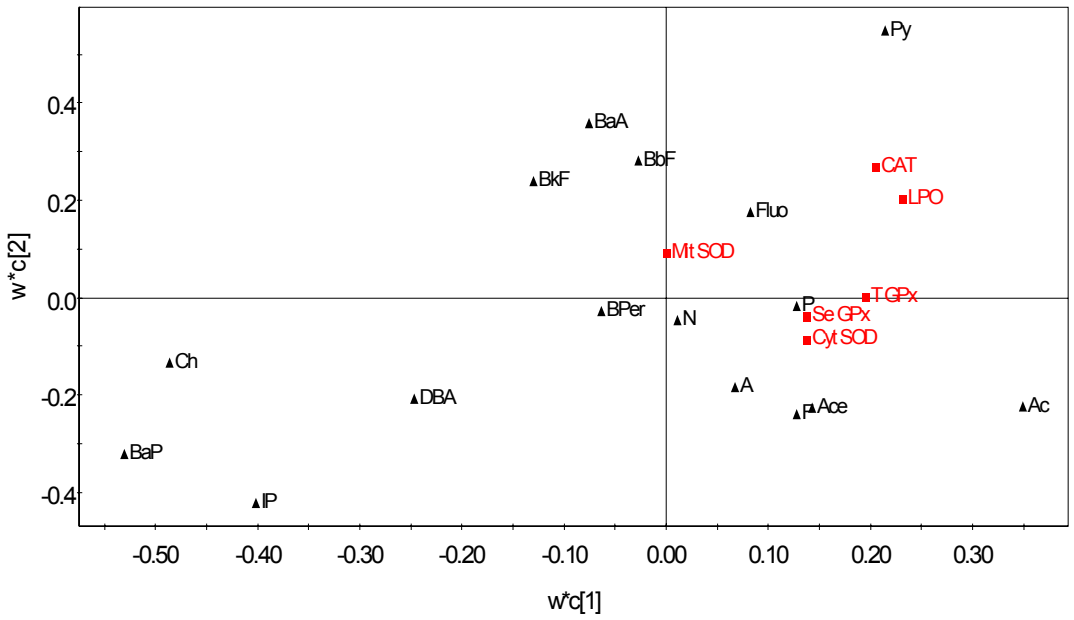


Figure 4.8 – PLS weights plot of the individual PAHs proportions (X-variables) and the stress related parameters (Y-variables) (n = 65).

The most relevant, in this case, are the PAH mixtures enriched in chrysene (Ch) and benzo[*a*]pyrene (BaP), which have the greatest loading factors. These variations do not mean that these PAH signatures are the responsible for the enzymes activity variation by itself. Instead, this activity variation might be due to some other factor that is associated with these PAH signatures.

4.4. DISCUSSION

This work intended to study the spatial and seasonal variation of oxidative stress (activity of antioxidant enzymes and lipid peroxidation) in a natural population of *R. decussatus* from several sites of the Ria Formosa lagoon affected by different PAH concentrations.

The levels of the antioxidant enzymes (SOD, CAT and GPx) activity in *R. decussatus* were of the same order of magnitude as other molluscs, namely, *Mytilus galloprovincialis*,

Ostrea edulis, *Cassostrea gigas* and *Tapes semidecussata*, collected at farms in the Ebro Delta, Spain (Solé *et al.*, 1994) and similar to those of *R. decussatus* from similar sites in the Ria Formosa (Geret *et al.*, 2003) (see Table 1.8 in Chapter 1 for further details).

The subcellular distribution of SOD in the clams digestive gland was in agreement with other studies, in which the most abundant form of SOD was the Cu/Zn-SOD found in the cytosol (Livingstone *et al.*, 1992; Orbea *et al.*, 2000; Geret *et al.*, 2003). In this study, cytosolic SOD (Cu/Zn-SOD) was on average 70% of the total SOD activity, which is similar to other results for the same species collected from the same area (Geret *et al.*, 2002a; Geret & Bebianno, 2004).

Significant spatial monthly variation exist for both Mit and Cyt SOD activities in the clam digestive glands (Figure 4.1). However, in each month, a different spatial distribution occurred making it impossible to elect which clams of a particular site or group of sites present higher or lower Mit or Cyt SOD activities. Seasonally, Mit SOD activity, in *R. decussatus* digestive gland was induced in the summer (except for July) and lowered in winter and spring. On the contrary, Cyt SOD activity was higher in autumn (October) and lower in the summer. Thus, Mit and Cyt SOD in the digestive gland are probably responding differently to the environmental conditions, to which clams are exposed to. SOD is often indicated as playing a central role in antioxidant defence. PAHs metabolism by the MFO system is known to originate metabolites capable of undergoing redox cycling, leading to superoxide anion generation (Lemaire *et al.*, 1994). Therefore, an increase in this enzyme activity would be expected as a response to an increase in PAH concentrations in the tissues. However, no relationship exists between SOD activities in the digestive gland and PAHs concentration in *R. decussatus* tissues (Table A6 in Annexe; Figure 4.6). A direct relationship between PAHs accumulation and SOD activity occurred in mussels, *Mytilus sp*., from the Mediterranean Sea (Porte *et al.*, 1991) and in *M. galloprovincialis* collected between

February and June in the Ebro Delta (Spain) (Solé *et al.*, 1995). However, when mussels *M. edulis* were used to assess the effect of an oil spill in the Galician coast (North-western coast of the Iberian Peninsula) no differences were observed in SOD activity along a PAH pollution gradient (Solé *et al.*, 1996). Similarly, no differences in SOD activity were observed in *M. galloprovincialis* with different PAH and PCB body burdens collected in different places from two Basque estuaries: the Bay of Biscay and Plentzia (Orbea *et al.*, 2002) and in the Venice lagoon (Livingstone *et al.*, 1995). The lack of SOD induction may point to the existence and efficiency in oxyradical scavenging by non-enzymatic dietary antioxidants, as vitamins E and C, glutathione, carotenoids or flavonoids, which constitute also important antioxidant defences (Halliwell, 1996; Lesser, 2006).

CAT normally acts on the H_2O_2 produced in the reduction of $O_2^{-\bullet}$. Since reactive oxygen species (ROS) may be produced in the PAHs metabolism, an induction of this enzyme after PAHs exposure was expected. Indeed, increases in CAT activities were observed in mussels *M. galloprovincialis* fed with benzo[*a*]pyrene contaminated food (50 mg/kg dry weight mussel) (Akcha *et al.*, 2000) and clams *Mercenaria mercenaria* transplanted to a PAHs contaminated site (9007.7 ng/g in the sediment) (Nasci *et al.*, 1999). However, because no induction of SOD was observed in this species, no induction of CAT should be expected. On the contrary, CAT activity was negatively related with the clam PAH content (Table A7 in Annexe; Figure 4.6). Such a decrease, associated with the absence of response from SOD, was also observed in mussels *Perna viridis* transplanted from a relatively clean site to various polluted sites in Hong-Kong (Cheung *et al.*, 2001). CAT activity, in *R. decussatus* digestive gland, presented both seasonal and spatial variations (Figure 4.2). Seasonally, CAT activity was highest in spring (March). Higher CAT activities in the same season were also reported in *Mytilus sp.* from the Biscay Bay (Cancio *et al.*, 1999; Orbea *et al.*, 1999), Cork Harbour (Power & Sheehan, 1996) and Barcelona (Solé *et al.*, 1995). However, the increase in CAT

activity was linked with the excess of oxyradicals arising from an increase in the metabolic rates observed during this season rather than contaminants exposure (Orbea *et al.*, 1999; Viarengo *et al.*, 1991). In the present study, the increase of CAT activity in spring (March) and decrease in the summer (August) was followed by a similar response in lipid peroxidation in the cells (Figure 4.5), pointing to an increase in oxidative stress. This is in agreement with the seasonal pattern of assimilation rates in *R. decussatus* from the Mundaka Estuary (Basque Country, North Spain), which is highest in March-July, associated with optimal nutritional conditions and increase in water temperatures (Urrutia *et al.*, 1999). Similar increases in CAT activity and LPO were also observed in *M. galloprovincialis* from the Biscay Bay (Cancio *et al.*, 1999). The negative relationship between CAT activity and PAH concentrations may also be related to seasonal factors. The rate of CAT turnover is known to be reduced by stressful conditions such as extreme high temperatures (Lesser, 2006). In the Ria Formosa, in summer, the water temperature may reach 30ºC during ebb tides (Chícharo & Chícharo, 2001a). In fact, the lowest CAT activities were in the summer (August I and II – Figure 3), coinciding with the highest PAH tissue concentrations thus leading to an apparent negative relationship between both. Additionally, the spatial variation indicated greater CAT activity in the digestive gland of clams from site 6 (Figure4.2) which is consistent with previous results for the same species in the Ria Formosa (Geret *et al.*, 2003).

Both T and Se GPx in the clam digestive gland showed similar behaviour during the annual cycle (Figure 4.4 and 4.5); although presenting maximum and minimum activities in different months, they are interrelated (Table 4.1; Figure 4.6). A general decrease was observed in T GPx and Se GPx activities in the clam digestive gland with increasing PAH concentrations in the whole soft tissues (Table A8 in Annexe; Figure 4.6). Similarly, T and Se GPx activities were depressed in freshwater mussels, *Unio tumidus*, transplanted to PAH, DDT, PCB and trace metal contaminated sites (Cossu *et al.*, 2000). Similar decreases in T and

Se GPx activities were also observed in the gills of clams *R. decussatus* exposed to Cd (4, 40 and 100 μg l^{-1} – Geret *et al.*, 2002a) or Cu (0.5, 2.5 and 25 μg l^{-1} – Geret *et al.*, 2002b). Clams *T. philippinarum* transplanted to PAH and PCB contaminated sites in the Venice lagoon presented also decreased Se GPx (Nasci *et al.*, 2000). This decrease in GPx activities associated to higher PAH concentrations reveals a precarious state of the exposed clams associated with PAHs toxicity (Cossu *et al.*, 2000). GPx activity is dependent on GSH, which is oxidised to GSSG. A constant supply of GSH is maintained by the activity of glutathione reductase (GR), which regenerates GSH, with the expense of NADPH (Gamble *et al.*, 1995). GR may be inhibited by chemical stress, thus, leading to a lack of GSH reposition and reduced GPx activity (Cossu *et al.*, 2000).

Se GPx role in the cell is closely related to CAT since they act on the same substrate. However, Se GPx affinity for H_2O_2 is much higher than catalase (Chaudière & Ferrari-Iliou, 1999). This is supported by the positive relationship between Se GPx and CAT activities in the clam digestive gland (Table 4.1). Thus, it is probable that Se GPx is the main enzyme responsible for the reduction of H_2O_2. CAT will only protect the cells during a short period of time, when exposed to high H_2O_2 concentrations (Chaudière & Ferrari-Iliou, 1999).

Lipid peroxidation (LPO) in the clam digestive gland was greatest in winter (November and December – Figure 4.5). In December, LPO was positively related to clam PAH body burden (Figure 3.1 and 4.5). Therefore, it is possible that clams are more sensible to oxidative stress induced by PAHs in the winter, which is also supported by the elevated CAT activity in December. However, when the whole data set was considered (all months), LPO in the clam digestive gland was inversely related to the PAH concentrations in the clam tissues, indicating that the increases in PAH concentrations in the clam tissues were not leading to membrane oxidative damage in *R. decussatus* digestive gland.

The seasonal variation in antioxidant enzyme activities and LPO may be related to several factors other than PAHs. Increases in water temperature may enhance accumulation and toxicity of contaminants, as well as biomarker response (Fisher *et al.*, 1999; Vidal *et al.*, 2002a). There were large water temperature variations between winter and summer (from 15 to 28ºC) during the course of this work (see Table A1 in Annexe) similar to those reported in other studies in the Ria Formosa lagoon: from 12 to 27ºC in winter and summer, respectively and also during the day (Δ6ºC in the summer) (Newton & Mudge, 2003). However, *R. decussatus*, from the Ria Formosa lagoon, seem to be well adapted to these variations, since their respiration and excretion rates are temperature independent in the range 20 – 32 ºC (Sobral & Widdows, 1997).

The reproductive cycle is also seasonal (Urrutia *et al.*, 1999). Somatic growth in the spring and gonadal development in early summer were observed in clams *R. decussatus* from the Mundaka Estuary in Urdaibai (Basque Country, North Spain) (Urrutia *et al.*, 1999). Vidal *et al*. (2002b) also reported increased CAT activities associated with the reproductive cycle in clams *Corbicula fluminea* from Sanguinet Lake (Southwest France). Enhanced activities of SOD, CAT and LPO levels were also observed in mussels *P. perna* from Ratones Grande Island (Brazil), in early summer (December) associated with the reproductive cycle (Wilhelm-Filho *et al.*, 2001). Thus, the seasonal variation observed in CAT activity in this study could also be related to the *R. decussatus* reproductive cycle.

Antioxidant enzymes activity and LPO may also be modulated by environmental contaminants other than PAHs. Geret *et al.* (2002), reported several changes in the Mit and Cyt SOD, CAT, T GPx, Se GPx activities and LPO in *R. decussatus* exposed to Cd, Cu and Zn (Geret *et al.*, 2002a ;Geret *et al.*, 2002b; Geret & Bebianno, 2004). These changes included induction and/or inhibition of the enzyme activities and LPO. Nasci *et al.* (1998) observed a negative relationship between SOD activity and Zn, DDT and PCB concentrations

and between CAT and Cr and Hg in *M. galloprovincialis* from the Venice lagoon. Enhanced CAT activities were observed in clams *Chamaelea gallina* exposed to PCBs (Aroclor 1254, 50 ppm) in water (Rodríguez-Ariza *et al.*, 2003).

In summary, antioxidant enzyme activities and LPO in the digestive gland of the clam *R. decussatus*, vary seasonally, but that seasonality was not related (Mit and Cyt SOD) or was negatively related (CAT, T GPx, Se GPx and LPO) to the clam PAH body burden. Though the decreased T and Se GPx activities may indicate a precarious state of clams, CAT activity is probably more related to temperature and clam reproductive cycle rather than with PAH concentrations. Also, the seasonal variation in the antioxidant enzymes could be the result of the synergistic effect of several contaminants and environmental conditions in the Ria Formosa throughout the year. Therefore other detailed experiments are needed to clarify the effect of PAHs in this clam species.

5. POLYCYLIC AROMATIC HYDROCARBONS ACCUMULATION AND RELATED OXIDATIVE STRESS BIOMARKERS IN A TRANSPLANT EXPERIMENT IN THE RIA FORMOSA LAGOON

5.1. INTRODUCTION

Invertebrates, especially bivalve molluscs such as mussels (*Mytilus edulis* and *M. galloprovincialis*) and clams (*Tapes philippinarum* and *R. decussatus*), which are filter-feeder and suspension-feeder bivalves are useful "biomonitor" species due to their bio-accumulation capacity of environmental contaminants (Nasci *et al.*, 2000; Bebianno & Serafim, 2003; Bodin *et al.*, 2004). The amount of contaminants concentrated in their tissues is not, however, enough to predict the "effect" of such compounds in aquatic life (Solé, 2000a). Thus, several studies have highlighted the importance of using an integrated approach where the accumulation of contaminants and their effects on organisms are measured to assess environmental quality (Livingstone *et al.*, 1995; den Besten, 1998; Nasci *et al.*, 2000; Solé, 2000a). Differences in natural variables (i.e. population variation, temperature, salinity, etc.) made the identification of any biological responses to toxic substances difficult to interpret. In such cases, the relocation of organisms from one site to another reduces the natural variability (Nasci *et al.*, 2000).

Caging techniques have been highly developed in recent years (Da Ros *et al.*, 2002; Roméo *et al.*, 2003a). These techniques have several advantages, such as the genetic uniformity of individuals from the same population and the possibility of placing caged organisms at sites where they are not naturally present. This procedure is considered an active biomonitoring of a specific environment (Nasci *et al.*, 1999; Roméo *et al.*, 2003b).

In this perspective, an active biomonitoring experiment was performed with clams transplanted between two sites (sites 5 and 7; Figure 2.1). Although, no spatial differences in the overall sediment and clams PAH concentrations were found, site 7 presented significantly higher sediment and clam PAH concentrations than site 5 in the winter and spring (see Chapters 2 and 3).

Several authors underlined the importance of measuring several biomarkers at the same time in the same organisms, to evaluate the effects of environmental contaminants (Porte *et al.*, 1998; Cajaraville *et al.*, 2000; Roméo *et al.*, 2003a; Bodin *et al.*, 2004). In this way, a multi biomarker approach was used in addition to the oxidative stress related parameters mentioned in Chapter 4. Other biomarkers such as benzo[*a*]pyrene hydroxylase (BPH) and glutathione S-transferases (GSTs), which are involved in the metabolism of xenobiotic organic compounds (see Chapter 1), were also measured. BPH is a cytochrome P450-dependent enzyme involved in the metabolisation of planar hydrophobic contaminants. Its activity has often been assayed to indicate CYP1A (a terminal component of the MFO system) activity in fish and molluscs (Shaw *et al.*, 2004). The GSTs (EC 2.5.1.18) form a group of multifunctional enzymes catalysing the conjugation of a broad range of electrophilic substrates, generally produced during the phase I of xenobiotics metabolism, to glutathione (Wilce & Parker, 1994). Therefore, they are involved in the cellular detoxification and excretion of many physiological and xenobiotic substances such as PAHs, PCBs and DDE (Hoarau *et al.*, 2001; Hoarau *et al.*, 2004). BPH enzymatic activity has been studied as an exposure marker of organic xenobiotics in mussels and clams (Michel *et al.*, 1994; Nasci *et al.*, 1999; Peters *et al.*, 1999; Nasci *et al.*, 2000; Porte *et al.*, 2001). Increased BPH activity was observed in mussels *M. galloprovincialis* exposed to benzo[*a*]pyrene (50 mg/kg dry weight) (Akcha *et al.*, 2000) and in clams *Mercenaria mercenaria* transplanted to PAH contaminated environments in Tampa Bay (Nasci *et al.*, 1999). Since GSTs can be induced or inhibited by organic xenobiotics, they have also been proposed as potential biomarkers of PAHs exposure. In fact, increases in GST activity were demonstrated in *R. decussatus* as a result of exposure to benzo[*a*]pyrene (1.5 and 3 µg l^{-1}) (Hoarau *et al.*, 2001). Besides their use as biomarkers of PAHs exposure, the determination of these enzymes activity will give an indication of the mechanism of metabolisation of PAHs by the clams.

In this transplant study, enzymatic activity (SOD, CAT, T GPx, Se GPx, BPH and GST) and LPO were determined both in the gills and in the digestive gland, and related with PAH concentrations accumulated in the clam whole soft tissues. Although the digestive gland is considered the organ where most of the PAHs metabolisation occurs (Livingstone *et al.*, 1985; Solé *et al.*, 1994), some authors have also highlighted the importance of the gills in the uptake and excretion of xenobiotics (Andersson & Förlin, 1992; Fossi *et al.*, 2000).

The measurements of chemical and biochemical parameters in this transplant experiment will contribute to increase the knowledge of the mechanism of PAHs accumulation and excretion and their effects in *R. decussatus* living in realistic environmental conditions.

5.2. MATERIALS AND METHODS

5.2.1. Experimental design

300 clams of similar shell length (2.5 – 3 cm) were collected in March 2004, from site 5 and net bags (50 x 80 cm), containing 30-40 individuals per bag, were placed at site 7 (see Figure 2.1 for site location). The bags were buried in the sediment and clams maintained separated from each other as in their natural farm environment.

The transplant experiment proceeded in two phases. During the first phase, one bag containing 30-40 clams was collected after 0, 1, 3, 7, 14 and 28 days. In the second phase, the remaining bags were back transplanted to their original location (site 5), and collected after 35, 42, 50 and 56 days. At the same time, 30 individuals were also collected at site 5 and were used as experimental control.

5.2.2. Physiological status of transplanted organisms

The condition index was measured on 15 animals for each sample, using the ratio of the weight of the soft tissue to the total weight (shell + soft tissues + palleal liquid) of the clam, multiplied by 100.

The mortality among the transplanted clams was less than 1%.

5.2.3. Tissue preparation

After collection, organisms were kept at 4ºC prior to their arrival at the laboratory. Immediately after arrival at the laboratory, clams were dissected and the gills and digestive glands excised. Samples were frozen in liquid nitrogen immediately after dissection and preserved at –80ºC until processed.

Tissues were homogenised in pools of two individuals (in order to have enough tissue to perform all the analyses) in a 20 mM Tris-HCl buffer (pH 7.6) containing 1 mM EDTA, 0.5 M sucrose, 0.15 M KCl and 1 mM dithiothreitol, using an Ultra-Turrax T 25 homogeniser (at 4ºC in an ice bath). The homogenates were centrifuged at 500 g for 15 minutes to remove rests of non-homogenised tissues and the resulting supernatant was centrifuged at 12,000 g, for 45 min, to obtain the mitochondrial fraction (pellet). The resulting supernatant was centrifuged at 100,000 g for 90 minutes (at 4ºC) to separate the microsomal (pellet) and cytosolic fractions (supernatant).

The mitochondrial fractions were resuspended in the homogenisation buffer, re-centrifuged for washing and kept at 4ºC overnight without resuspension. Immediately before analysis they were resuspended in 1 ml of homogenisation buffer.

The microsomal and cytosolic fractions were kept at -80ºC without resuspension until analysis. Immediately before analysis microsomes were resuspended in 10 mM Tris-HCl pH 7.6

(containing 20% v/v glycerol, 1 mM dithiothreitol and 1 mM EDTA) to give a total protein concentration of approximately 6 mg ml^{-1}.

Total proteins and LPO levels were analysed in the gills and digestive gland of a different set of organisms due to the interference of sucrose on the protein assay by the Lowry method (Lowry *et al.*, 1951). After dissection, tissues of 5 organisms were homogenised separately in 20 mM Tris-HCl buffer (pH 8.6) containing 150 mM NaCl and centrifuged at 3000 g for 15 min (at 4ºC). The analyses were carried out in the supernatant.

All reagents were purchased from SIGMA with the exception of phosphates that were purchased from Merck.

5.2.4. Chemical analysis

PAH concentrations were determined in three pools of three whole clam tissues of each sample. The analytical method used is described in section 3.2.2.

5.2.5. Biochemical Analyses

The activities of SOD and CAT were measured in the mitochondrial and cytosolic fractions of gills and digestive glands of five organisms as described in Chapter 4.

T and Se GPx activities were measured in the cytosolic fractions of gills and digestive glands of five organisms as described also in Chapter 4.

LPO was determined in the whole homogenate of gills and digestive glands of five organisms as described also in Chapter 4.

5.2.6. Benzo[*a*]pyrene Hydroxylase

BPH activity was determined fluorometrically at 25°C as described in Livingstone *et al.* (1985). All assays were carried out in duplicate at 25ºC. Tubes containing, at a final volume of 1 ml, 50 mM Tris-HCl pH 7.6 buffer, 0.2 mM NADPH and approximately 1 mg of microsomal protein were gently shaken. The reaction was initiated by the addition of benzo[*a*]pyrene in dimethylformamide (60 mM final concentration) and stopped by the addition of 1 ml of ice-cold acetone. Tubes were centrifuged (3000 g × 10 min) and an aliquot of the supernatant mixed with 2.3 times its volume of 8% triethylamine and centrifuged as before. The fluorescence of the resulting supernatant was measured using an excitation wavelength of 467 nm and the emission measured at 525 nm. Results are described in terms of arbitrary units of fluorescence produced over unit of time. The reaction was stopped after 0 and 10 min and duplicate tubes were run. Microsomal protein was measured by the method of Lowry *et al.* (1951).

5.2.7. Glutathione S-transferase

GST activities were measured spectrophotometrically at 340 nm in the cytosolic fractions by following the conjugation of the acceptor substrate 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (Habig *et al.*, 1974). The reaction mixture contained 85 mM KH_2PO_4/K_2HPO_4 (pH 6.5), 1 mM CDNB, 10 mM GSH and 10 µl of cytosolic fraction in a 1 ml final volume. Reaction was started by the addition of GSH.

Results are expressed as the formed conjugate per minute per milligram of total protein.

5.2.8. Statistical Analysis

Chemical and biochemical data are expressed as mean ± standard deviation. Parametric one-way analysis of variance test with *post-hoc* comparisons was used to test differences

between groups when homogeneity of variances (Levene's test) and normality (Shapiro Wilk test) prevailed. In other cases, nonparametric Kruskall-Wallis and Mann-Whitney U-test were applied.

Principal Components Analysis (PCA) was used to investigate the relationships between variables and discriminate between control and transplanted clams collected at different times. The mean values were used.

The relationship between the enzymes activity and the PAH concentrations in clam tissues was analysed by Spearman Ranks Correlation (for non-parametric data) and PLS analysis. In PLS analysis, the PAH concentrations or proportion data were used as an X-block and the stress related markers as the Y-block. PLS analysis results in model coefficients for the variables, called PLS-weights. The weights for the X-variables, denoted w, indicate the importance of these variables, how much they "in a relative sense" participate in the modelling of Y. The weights for the Y-variables, denoted by c, indicate which Y-variables are modelled in the respective PLS model dimensions. When these coefficients are plotted in a so-called "wc" plot, a picture showing the relationships between X and Y is obtained.

Significant differences and correlations were considered when $p<0.05$.

5.3. RESULTS

5.3.1. Physiological status

Table 5.1 shows the mean (±SD) condition index of clams at site 5 and transplanted to site 7. The mean condition index (CI) of the clams at site 5 ranged between 19.5 (day 28) and 23.1 (day 3). The CI significantly changed in the first 3 days, after what it remained unchanged. The same occurred in the transplanted clams after 1, 3 and 14 days of transplantation. No significant differences were observed in the CI of the clams between the two sites ($p>0.05$).

Table 5.1 – Condition Index (mean ± SD) of the clams

Sampling Day	Site 5	Transplanted to site 7
0	21.5 ± 1.2	21.5 ± 1.2
1	20.2 ± 1.2*	20.4 ± 0.9*
3	23.1 ± 1.6*	22.9 ± 0.8*
7	20.9 ± 1.2	20.7 ± 1.8
14	20.8 ± 1.2	22.6 ± 1.3*
28	19.5 ± 2.8*	20.8 ± 1.5
		Backtransplanted from site 7 to site 5
35	21.1 ± 2.3	20.6 ± 1.6
42	21.4 ± 2.0	20.5 ± 1.0
50	20.1 ± 2.6	20.9 ± 1.8
56	19.6 ± 2.0	20.8 ± 1.4

* Significantly different from day 0 ($p<0.05$)

When clams were backtransplanted from site 7 to site 5, the CI of both clam populations was similar ($p>0.05$).

5.3.2. PAH concentrations

A. Transplant Experiment

PAH concentrations in the clams from site 5 and transplanted to site 7 are presented in Figure 5.1 and Table A10 in Annexe.

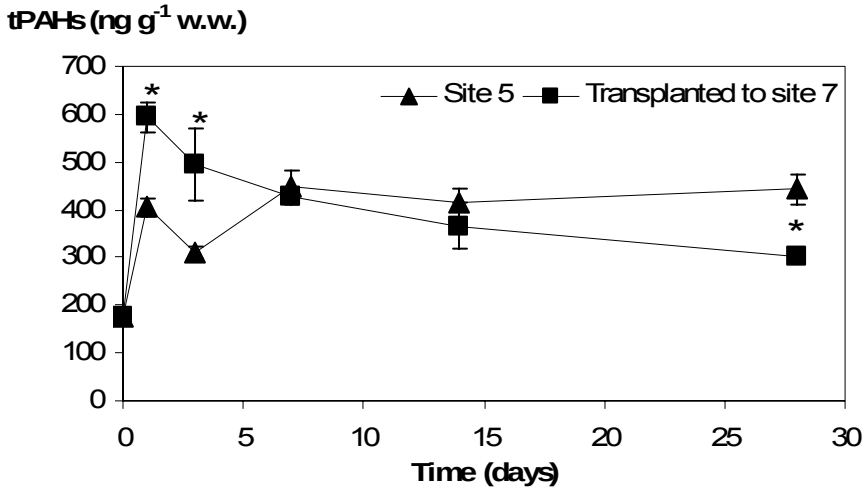


Figure 5.1 – Variation (mean ± SD) of tPAH concentration (ng g^{-1} w.w.) in the whole soft tissues of the clams from site 5 and transplanted to site 7. Significant differences between sites are marked with *.

tPAH concentrations significantly increase on the first day in clams from both sites (Figure 5.1) but this increase was significantly higher in the transplanted clams (594±32 ng.g^{-1}) (p<0.05). Afterwards, tPAH concentrations in the clams from site 5, significantly decreased until day 3 and increased again until day 7. However, from day 7 until the end of the experiment, tPAH concentrations were significantly higher than at the beginning of the experiment (day 0) but not different from day 1 (p>0.05). In clams transplanted to site 7, however, tPAH concentrations decreased logarithmically after day 1 (log tPAH = 2.73 – 9.90 x 10^{-3}t; r = -0.890; n = 15; p<0.05), reaching levels (304±16 ng.g^{-1}) significantly lower than those from site 5 in the end of the experiment but significantly higher when compared to those at the beginning of the experiment (p<0.05).

Figures 5.2 and 5.3 present the variation of PAH concentrations as a function of aromatic ring numbers and of individual PAHs. The accumulation of the 2+3 ring PAHs was 37% of tPAH accumulated in clams from site 5 and around 48% in clams transplanted to site 7. The variation pattern of the 2+3 ring PAHs with time was similar to that of tPAH in both clam groups (Figure 5.1).

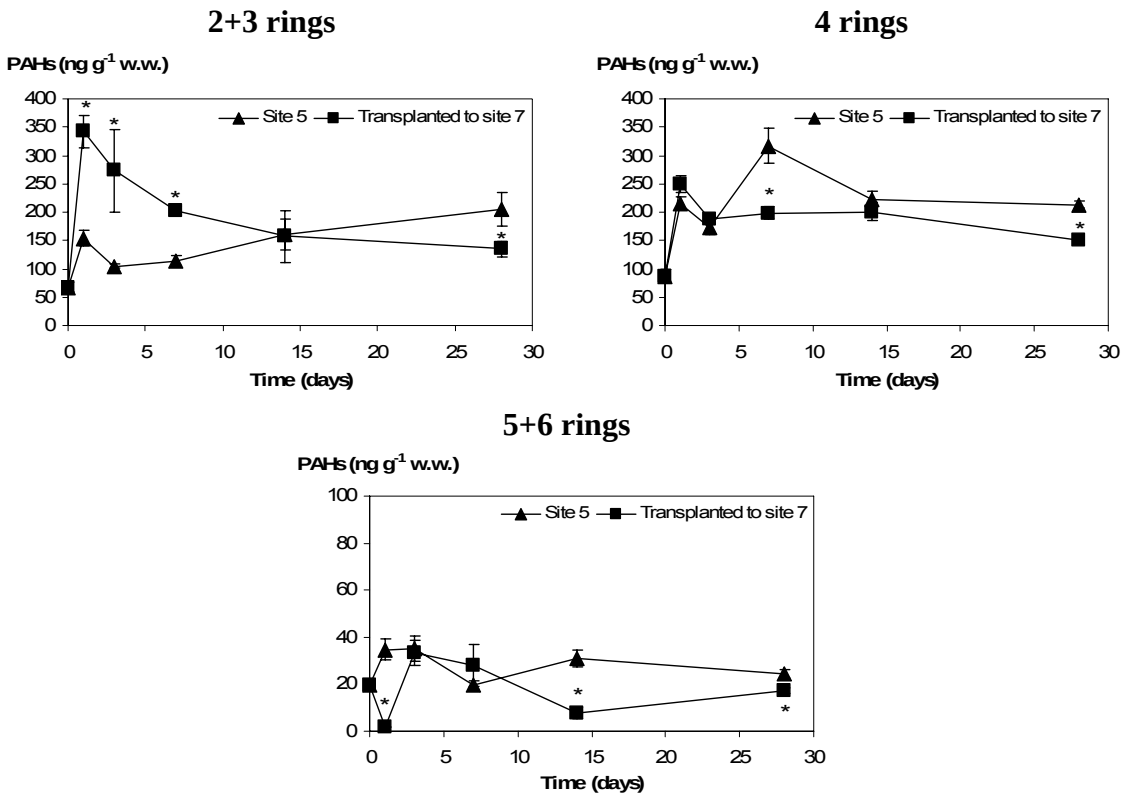


Figure 5.2 – Variation (mean ± SD) of PAH concentrations (ng g^{-1} w.w.) according to aromatic ring number in the whole soft tissues of the clams from site 5 and transplanted to site 7. Significant differences between sites are marked with *.

In clams from site 5, the 2+3 ring PAH concentrations increased significantly at day 1 decreasing until day 3 (p<0.05). Between days 3 and 28, a linear increase was observed (2+3 ring $PAH_{Site\ 5}$ = 92.22 + 4.16 t; r = 0.934; n = 12; p<0.05) and by the end of the experiment levels were significantly higher than at day 0. In clams transplanted from site 5 to site 7, the 2+3 ring PAH concentrations increased also significantly at day 1 and, like tPAH (Figure 5.1), to values significantly higher than at site 5. Afterwards 2+3 ring PAH concentrations decreased logarithmically (log 2+3ring $PAH_{Transplanted}$ = 2.46 – 0.0138 t; r = - 0.834; n = 15; p<0.05) reaching by the end of the experiment significantly higher concentrations than at the beginning of the transplant experiment (day 0).

The major individual PAH in this group responsible for the differences at both sites was acenaphthene (Ac) that was around 70% of the 2+3 ring PAH concentrations in clams from site 5 and around 80% in transplanted clams (Figure 5.3). The variation of Ac followed a similar

pattern to that of tPAH (Figure 5.1) and 2+3 ring PAHs (Figure 5.2). Ac levels significantly increased after 24 hours in clams from both sites but were significantly higher in transplanted clams (248 ng g^{-1} d^{-1}). Afterwards, Ac concentration decreased in clams from site 5 until day 7 increasing then linearly until day 28 when it reached its highest levels ($Ac_{site\ 5}$ = 18.73 + 5.33 t, r = 0.944; n = 9; p<0.05). In transplanted clams, however, Ac concentrations continuously decrease after day 1 reaching, by the end of the experiment, significantly lower concentrations than in clams from site 5 but significantly higher than those at the beginning of the experiment (p<0.05). Fluorene (F) concentrations represented around 10% of the 2+3 ring PAHs in clams from site 5 and 6% in clams transplanted to site 7. In clams from site 5, F concentrations increased linearly until day 14 ($F_{site\ 5}$ = 3.85 + 1.46 t; r = 0.963; n = 15; p<0.05) decreasing afterwards until day 28 to levels significantly higher than at the beginning of the experiment (p<0.05). In transplanted clams, however, the highest F concentration was at day 7 after also a linear increase ($F_{Transplanted}$ = 6.04 + 1.89 t; r = 0.876; n = 12; p<0.05) decreasing also linearly afterwards ($F_{Transplanted}$ = 21.85 – 0.49 t; r = -0.792; n = 9; p<0.05) but, at the end of the experiment levels were similar to those at the beginning (p>0.05). Phenanthrene (P) and anthracene (A) concentrations were around 15% and 2% respectively, of the 2+3 ring PAHs accumulated in both clam groups. Their variation was also similar in clams from both sites with significantly higher P at day 1 in transplanted clams, reaching the highest concentrations at day 7. Afterwards, P concentrations decreased in clams from both sites.

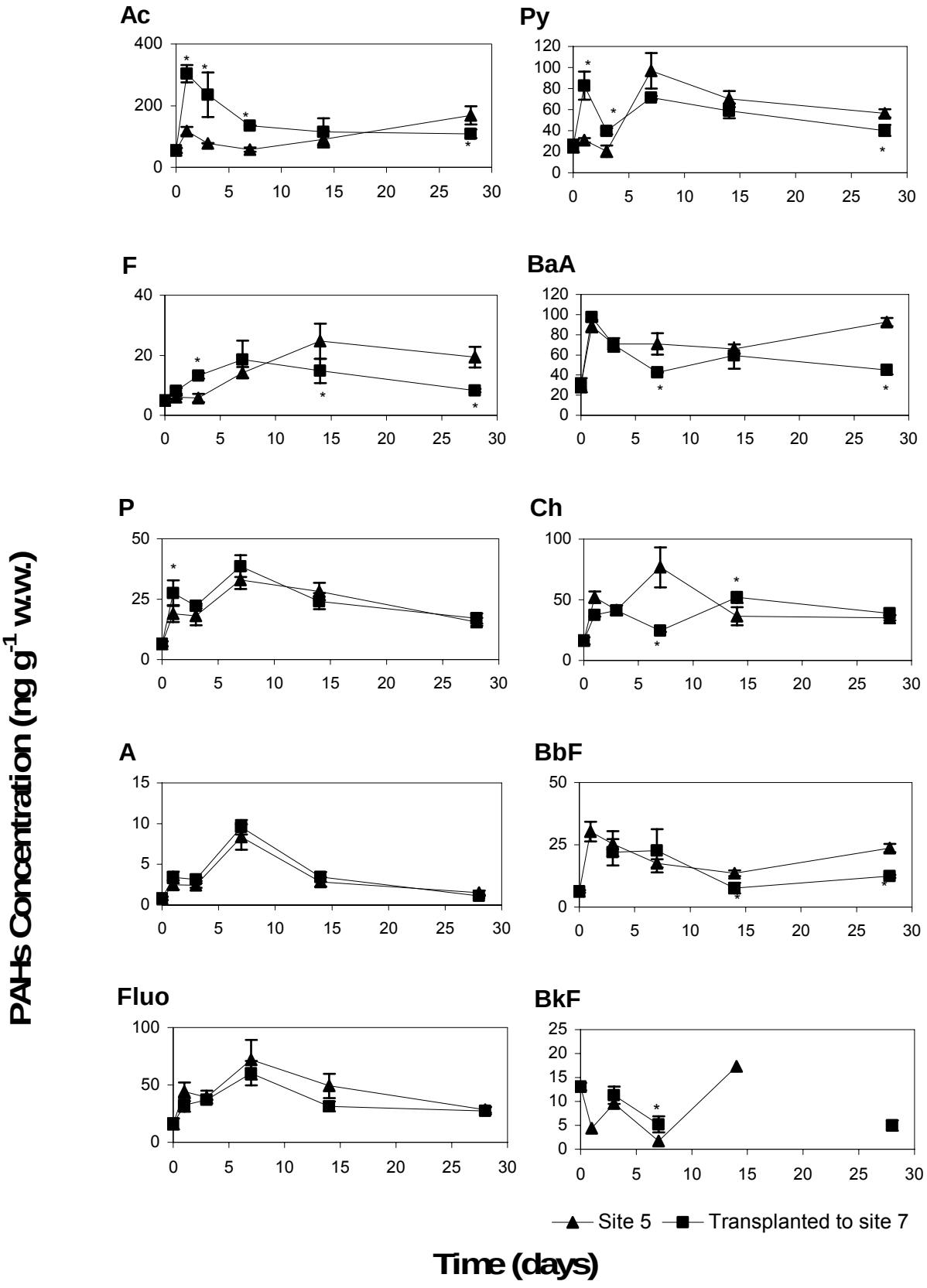


Figure 5.3 – Variation (mean ± SD) of individual PAH concentrations (ng g^{-1} w.w.), in whole soft tissue of the clams from site 5 and transplanted to site 7. Significant differences between sites are marked with *.

This decrease was linear for those at site 5 but not at site 7 ($P_{site\ 5}$ = 39.14 – 0.83 t; r = -0.957; n = 9; p<0.05). By day 28, P concentration was 47% less than at day 7 in clams from site 5 and 56% less in transplanted clams but still significantly higher than at the beginning of the experiment. Similarly to P, anthracene (A) concentrations increased linearly in clams from both sites (with similar accumulation rates) until day 7 ($A_{site\ 5}$ = 0.68 + 1.03 t; r = 0.944; n = 12; p<0.05; $A_{Transplanted}$ = 1.05 + 1.16 t; r = 0.953; n = 12; p<0.05), decreasing afterwards. By the end of the experiment, A concentrations decreased 18% in clams from site 5 and 12% in transplanted clams when compared to those at day 7. By the end of the experiment A levels were higher than at day 0 in clams from site 5 and lower in transplanted clams.

The variation of the 4 ring PAH concentration was similar in clams from both sites, except at days 7 and 28, when it was significantly higher in clams from site 5 (Figure 5.2). However, clams from site 5 accumulated at day 7 higher amounts of the 4 ring PAHs (around 2 fold) than that of the 2+3 ring PAHs, suggesting that the contribution of the former was more important to the variation of tPAH concentrations in clams at this site. In fact, in clams from site 5, the 4 ring PAHs group was around 55% of tPAH, while in transplanted clams it represented less than 50%. In clams from site 5, the 4 ring PAHs concentration increased significantly at day 1, decreasing afterwards, only to increase again reaching, by day 7, its highest value. Levels decreased once more by day 14 and remained unchanged until the end of the experiment (day 28). By this time, levels were still significantly higher than at the beginning of the experiment. In the transplanted clams, the 4 ring PAH concentrations, like in clams from site 5, also increased significantly on the first day but remained unchanged until the end of the experiment, at levels significantly higher than at the beginning. The most relevant individual PAHs among the 4 ring group in clams from site 5 were pyrene (Py) and benzo[*a*]anthracene (BaA) representing 25% and 34% of the 4 ring PAH concentrations respectively. In transplanted clams, the most relevant individual PAHs were also pyrene (Py) and BaA each representing around 30% of the 4 ring

PAHs. These PAHs, along with chrysene (Ch) were responsible for the major differences of 4 ring PAHs between sites since the variation pattern of fluoranthene (Fluo) was similar in clams from both sites and represented around 20% of the 4 ring PAH group. Fluo concentrations increased similarly in both clam groups until day 7 when the maximum concentration was reached. Such increase was linear for transplanted clams ($Fluo_{Transplanted}$ = 23.6 + 5.82 t; r = 0.798; n = 12; p<0.05) though not for those at site 5. Afterwards, it decreased in clams from both sites and by day 28, Fluo concentrations were 39% and 46% lower than at day 7 in clams from site 5 and in transplanted clams, respectively, but still significantly higher than at the beginning of the experiment. Contrary to Fluo, Py presented a dissimilar variation between clams from both groups with time. In clams from site 5, the highest Py concentration (97.0±16.8 ng g^{-1} w.w.) was reached after 7 days, opposite to the transplanted clams that reached the highest level (82.9±13.3 ng g^{-1} w.w.) at day 1 which remained unchanged until day 3. By the end of the experiment concentrations decreased 42% and 44% respectively, but only linearly for the transplanted clams ($Py_{Transplanted}$ = 80.53 – 1.46 t; r = -0.949; n = 9; p<0.05) reaching levels significantly higher than at day 0. Benzo[*a*]anthracene reached its highest concentration at day 1 in both clam groups: 88.5±6.1 and 97.3±4.1 ng g^{-1} w.w., respectively. However, by day 7, BaA concentrations were significantly lower in transplanted clams (p<0.05). This pattern was maintained until the end of the experiment (day 28) although BaA levels were significantly higher than at the beginning of the experiment in both clam groups. Chrysene (Ch) concentrations were more variable in clams from site 5 than in transplanted clams though it represented only 20% of the 4 ring PAHs in both clam groups. Similarly to P, A, Fluo and Py, the highest Ch concentrations in clams from site 5 were at day 7 (76.8±16.5 ng g^{-1} w.w.) 2-fold higher than in transplanted clams (p<0.05). Afterwards, it decreased around 54% until the end of the experiment to levels significantly higher than at the beginning. In transplanted clams, Ch concentrations fluctuated during the

course of the experiment and by day 28 were also significantly higher than at the beginning ($p<0.05$; Figure 5.3).

The 5+6 ring PAH concentrations were significantly lower (less than 10% of tPAH) than the other PAH ring groups ($p<0.05$; Figure 5.2). Although the variation of the 5+6 ring PAHs with time was different between the two clam populations, by the end of the experiment, their concentration was similar to those at the beginning though slightly lower at transplanted clams (Figure 5.2). Several individual PAHs of 5 and 6 aromatic rings were analysed (see section 3.2.2) however, only benzo[*b*]fluoranthene (BbF) and benzo[*k*]fluoranthene (BkF) data are presented since they were the most frequently detected in clam tissues (Figure 5.3). BbF and BkF concentrations varied similarly in the tissues of clams from both sites. Though accumulated at a lesser extent, the variation of BbF and BkF concentrations with time was similar to that of Ac and BaA in both clam groups. Its highest concentrations were at day 1 for clams at site 5 and transplanted clams (30.3±4.0 ng g^{-1} w.w. and 22.0±5.3 ng g^{-1} w.w., respectively). Afterwards, a 22% decrease in BbF concentrations was observed in clams from site 5, while in transplanted clams this decrease was more significant (45%) and only in this clam group BbF levels were similar to those at the beginning of the experiment. BkF presented an opposite variation relative to all the other PAHs, decreasing at days 1 and 7 and increasing at days 3 and 14 (Figure 5.3).

In summary, the most striking differences between PAH concentrations in clams from site 5 and those transplanted to site 7 occurred at days 1, 3, 7 and 28 (Figure 5.1). At day 1, the significantly higher accumulation was due to the 3 ring PAH Ac and the 4 ring PAH Py in the transplanted clams (Figure 5.3). At day 3 a significant decrease in these PAHs along with BaA and Ch occurred and with the exception of Ac and Py, levels were similar in clams at both sites (Figure 5.3). By day 7, the decrease in the 4 ring PAHs continued in transplanted clams but not in clams from site 5 where Py and Ch concentrations significantly increased while BaA concentrations remained unchanged (Figure 5.3). Consequently, at day 7 tPAH concentrations

were no longer different between clam groups (Figure 5.1). During the rest of the experiment, though most of the 4 ring PAH concentrations decreased in clams at both sites, the 3 ring Ac and the 4 ring BaA increased in clams from site 5 (Figure 5.3). Therefore, by day 28, tPAH concentrations were significantly higher in clams from site 5 than in those transplanted from site 5 to site 7 (Figure 5.1) due mainly to the 2+3 rings Ac and F, the 4 rings Py and BaA and the 5+6 rings BbF, which were significantly higher than at the beginning of the experiment.

In what concerns human health, BaP equivalents were calculated as described in section 3.2.2. Similarly to the clams previously collected at sites 5 and 7 during this time of year (January to May), clams were safe for consumption presenting BaP equivalents between 6 and 17 ng g^{-1} w.w.).

B. Backtransplant Experiment

Figure 5.4 shows the variation of PAH concentrations in clams from site 5 and those backtransplanted from site 7 to site 5 (see also Table A11 in Annexe). The variation of tPAH concentration with time was distinct between the two clam groups.

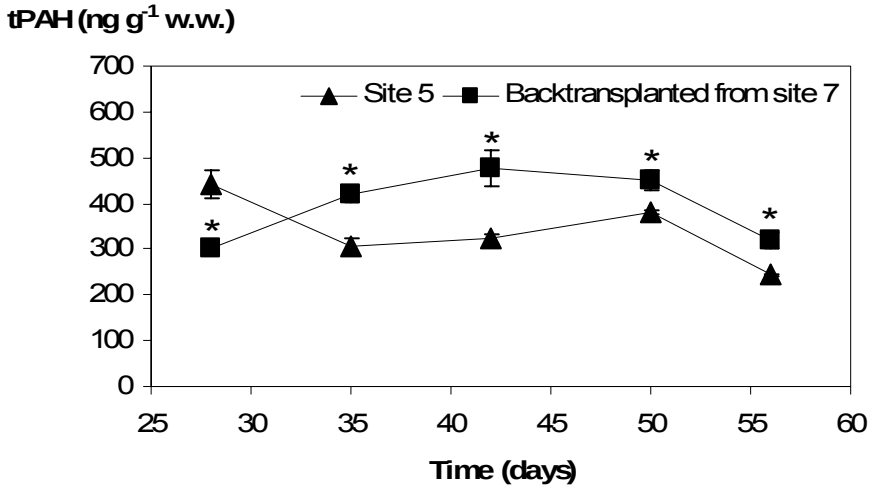


Figure 5.4 – Variation of tPAH concentration (ng g^{-1} w.w.) in the whole soft tissues of the clams from site 5 and those backtransplanted from site 7 to site 5. Significant differences between sites are marked with *.

In clams from site 5, tPAH concentrations significantly decreased from day 28 to day 35 ($p<0.05$), remaining unchanged until day 50 and decreasing again at the end of the experiment when the lowest concentration was reached.

In clams backtransplanted from site 7 to site 5, tPAH concentrations were significantly lower than in clams originally from site 5. In backtransplanted clams, tPAH concentrations significantly increased from day 28 to day 42 decreasing afterwards but reaching always significantly higher levels than in clams originally from site 5 until the end of the experiment ($p<0.05$).

Figures 5.5 and 5.6 present the variation of PAH concentrations as a function of aromatic ring numbers and of individual PAHs. The variation of the 2+3 ring PAHs group presents the greatest similarity to tPAH variation, in both clam groups. However, contrary to the transplant experiment this PAH group represented only 26 and 42% of the tPAH in clams from site 5 and backtransplanted clams, respectively. In clams from site 5, 2+3 ring PAH concentrations significantly decreased during the whole period. Oppositely, in clams backtransplanted from site 7 to site 5, the 2+3 ring PAH concentrations significantly increased until day 42 with a pattern similar to that of tPAH ($p>0.05$). Considering the individual PAHs (Figure 5.6), acenaphthene (Ac) was the most relevant PAH representing 73% of all 2+3ring PAHs accumulated in clams from site 5 and 80% in backtransplanted clams. In clams from site 5, Ac and fluorene (F) decreased similarly until day 35 after what levels remained unchanged, although Ac concentrations were 10 fold higher than F. In backtransplanted clams, similarly to tPAH (Figure 5.4) and 2+3 ring PAH variations (Figure 5.5) Ac concentrations increased until day 42 reaching significantly higher levels than in clams at site 5. Ac concentrations, decreased afterwards to their initial concentration (day 28) and by the end of the experiment were no longer different of clams from site 5.

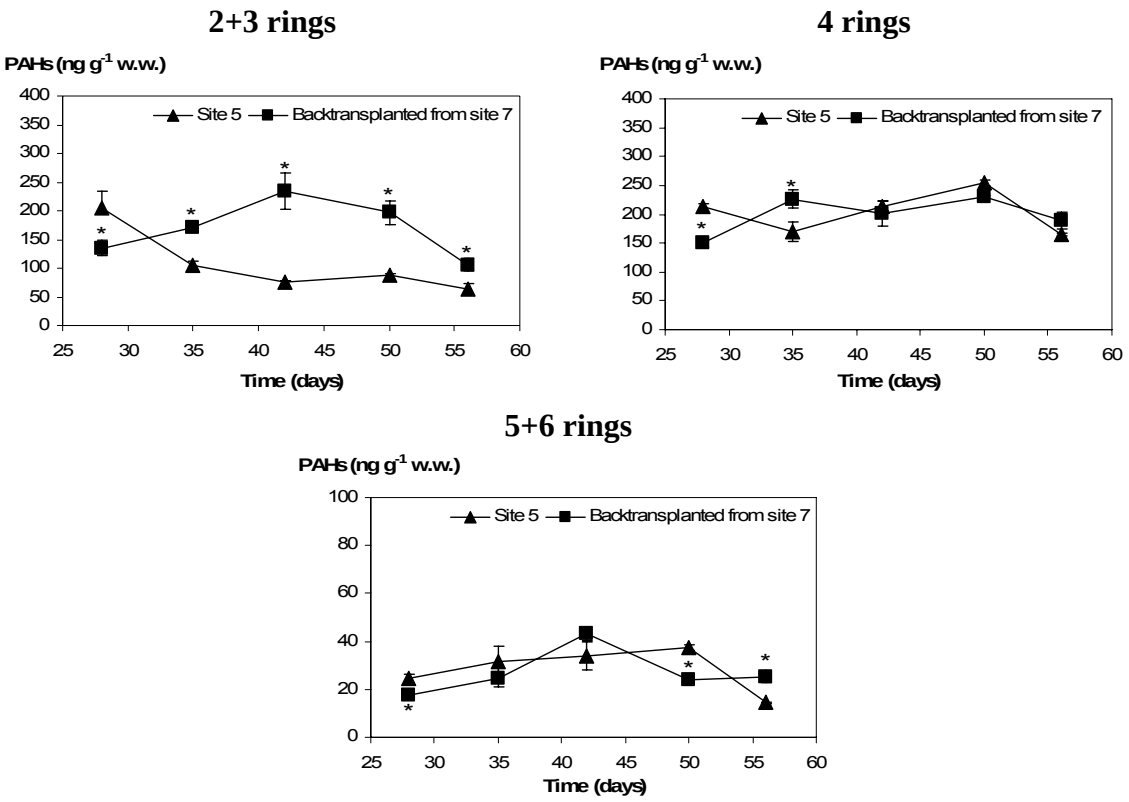


Figure 5.5 – Variation (mean ± SD) of PAH concentrations (ng g^{-1} w.w.) according to aromatic ring number in the whole soft tissues of the clams from site 5 and backtransplanted from site 7 to site 5. Significant differences between sites are marked with *.

The variation of fluorene (F) concentration was similar to that of Ac in clams from site 5 although in the backtransplanted clams the maximum concentration was at day 35 (significantly higher than in clams from site 5; $p<0.05$). However, F represented only 7% of the 2+3 ring PAHs in both clam groups. Phenanthrene (P) and anthracene (A) did not change with time in clams from site 5 and represented 18% and 2% of the 2+3 ring PAHs, respectively. In backtransplanted clams these PAHs represented 12% and 2% of the 2+3 ring PAHs, respectively and a maximum concentration occurred at day 35, for both PAHs significantly higher than that of clams from site 5.

The 4 ring PAHs group was the most relevant in the backtransplant experiment representing around 64 and 51% of tPAH in clams from site 5 and backtransplanted clams, respectively. The variation of the 4 ring PAH concentrations however depended on the clam groups. In clams from site 5, the 4 ring PAH concentrations significantly decreased until day 35,

increasing then linearly until day 50 (4 ring PAHs = -27.95 + 5.70 t; r = 0.950; n = 9; p<0.05). Afterwards, 4 ring PAH concentrations in clam whole soft tissues decreased again reaching significantly lower levels than at day 28. In the backtransplanted clams however, the 4 ring PAH concentrations fluctuated through the whole experiment and at day 56, PAH concentrations were no longer different from those at day 28 (Figure 5.5). Significant differences in 4 ring PAH concentrations between sites were observed only at the beginning of the backtransplant experiment and after one week (p<0.05).

In the 4 ring PAHs group, benzo[*a*]anthracene (BaA) was the most relevant individual PAH representing around 40% of the 4 ring PAHs in both clam groups (Figure 5.6). Fluoranthene (Fluo) did not change in the whole soft tissues and represented around 20% of the 4 ring PAHs in clams from both sites. That was not however, the case for pyrene (Py) and BaA: in clams from site 5, after an initial decrease (from day 28 to day 35), Py and BaA concentrations increased linearly until day 50 ($Py_{Site\ 5}$ = -3.11 + 0.78 t; r = 0.963; n = 9; p<0.05; $BaA_{Site\ 5}$ = -40.07 + 3.04 t; r = 0.972; n = 9; p<0.05) after what their concentrations decreased again. Despite the similarity in Py and BaA variations in clams from site 5, Py represented only 14% of the 4 ring PAHs.

In the backtransplanted clams, Py and BaA were accumulated between days 28 and 35. Afterwards, while Py concentrations decreased linearly ($Py_{Backtransplanted}$ = 73.01 – 0.79 t; r = -0.863; n = 12; p<0.05), BaA remained unchanged. Similarly to clams from site 5, Py represented only 18% of this PAH group. Significant differences between clam groups were only observed in the first week for Py and at day 50 for BaA (p<0.05). Chrysene (Ch) concentrations represented around 24% of the 4 ring PAHs and were always similar in clams from both groups. However, while a linear increase was observed until day 50 for clams at site 5 ($Ch_{Site\ 5}$ = -2.57 + 1.31 t; r = 0.957; n = 12; p<0.05), Ch concentrations remained unchanged in backtransplanted clams.

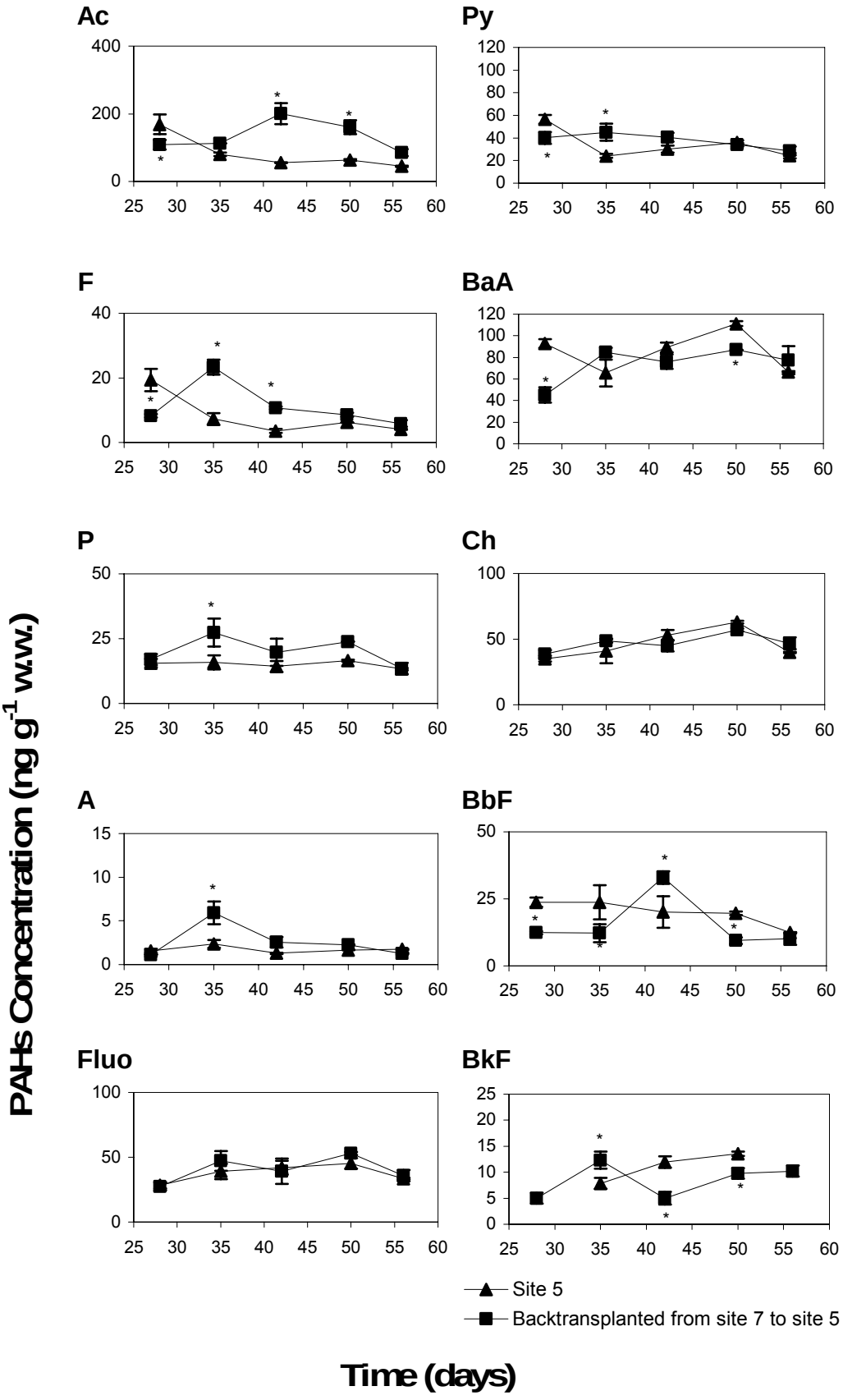


Figure 5.6 – Variation (mean ± SD) of individual PAH concentrations (ng g^{-1} w.w.), in the whole soft tissue of the clams from site 5 and backtransplanted from site 7 to site 5. Significant differences between sites are marked with *.

Similarly to the transplant experiment, the 5+6 ring PAHs were the least accumulated (less than 10% of tPAH) by both clam groups ($p<0.05$; Figure 5.5). In clams from site 5, the 5+6 ring PAH concentrations increased linearly until day 50 (5+6 ring PAHs = 10.00 + 0.56 t; r = 0.870; n = 12; $p<0.05$). By day 56, however, PAH concentrations decreased to levels significantly lower than those at day 28 ($p<0.05$). In the whole soft tissues of backtransplanted clams, the 5+6 ring PAH concentrations, like the 2+3 ring PAHs, significantly increased until day 42, and were significantly higher than in clams remaining at site 5. Their concentrations decreased afterwards to levels significantly lower than those in clams from site 5 and those at day 28 ($p<0.05$; Figure 5.6). Benzo[*b*]fluoranthene (BbF) concentrations decreased linearly during the time of the experiment in clams from site 5 ($BbF_{Site\ 5}$ = 35.60 – 0.37 t; r = -0.789; n = 15; $p<0.05$). However, in backtransplanted clams, a maximum was reached at day 42. Benzo[*k*]fluoranthene (BkF) was detected in the whole soft tissues of clams from site 5 only between days 35 and 50. During this period BkF concentrations increased continuously. In the backtransplanted clams, BkF concentrations increased in the first week, decreasing afterwards to the initial level. From day 42 to day 50, BkF concentrations increased again remaining until the end of the experiment at levels significantly higher than at day 28 (Figure 5.6).

In summary, during the backtransplant experiment (Figure 5.4) tPAH concentrations varied less and with a different pattern when compared to the transplant experiment (Figure 5.1). Such variation was mainly due to changes of Ac and BaA concentrations (the most accumulated 2+3 and 4 ring PAHs, respectively). More specifically, at day 35 the differences in tPAH concentrations between clam groups were the result of the preferential accumulation of F, P, A, Py, BaA and BkF by clams backtransplanted from site 7 to site 5 to levels significantly higher than in clams from site 5. In these clams (site 5), most of these PAHs concentration decreased (Ac, F, Py and BaA) or remained unchanged (P, A, Ch and BbF). As a result, tPAH concentrations significantly increased in backtransplanted clams and decreased in clams from

site 5 (Figure 5.4). At day 42, some of these PAHs concentration decreased in backtransplanted clams (F, P, A and BkF) to similar (P and A) or significantly lower (BkF) levels than in clams from site 5. However, Ac and BbF concentrations increased significantly in backtransplanted clams, causing tPAH concentrations to remain significantly higher than in clams from site 5 (Figure 5.4). Between days 42 and 56, most PAH concentrations decreased in backtransplanted clams (Ac, F, P, A, Py and BbF), remaining unchanged (Ac, F, P, A, Fluo and Py) or decreasing (BaA, Ch and BbF) also in clams from site 5. Thus, a general decrease was observed in tPAH concentrations in both clam groups although, by the end of the experiment tPAH concentrations in backtransplanted clams were still significantly higher than clams from site 5 (Figure 5.3).

As in the transplant experiment, BaP equivalents were generally low (between 6 and 18 ng g^{-1} w.w.) and clams were safe for consumption.

5.3.3. Biochemical Parameters

A. Transplant Experiment

The levels of antioxidant enzyme activities in clams from site 5 and transplanted to site 7 are in Figure 5.7 (see also Table A12 in Annexe). Most antioxidant enzymes exhibit opposite trends between tissues and activities were in most cases higher in the gills than in the digestive gland. In general, the variation of antioxidant enzyme activities was similar at both sites except for CAT, T GPx and Se GPx in the gills and Cyt SOD and T GPx in the digestive gland.

SOD activity was measured in both the mitochondrial (Mit SOD) and cytosolic (Cyt SOD) fractions of *R. decussatus* gills and digestive glands and was mainly found in the cytosolic fraction (70 – 94% in the gills and 84 – 94% in the digestive gland). The variation of Mit SOD activity in the gills, was similar in clams from both sites (p>0.05).

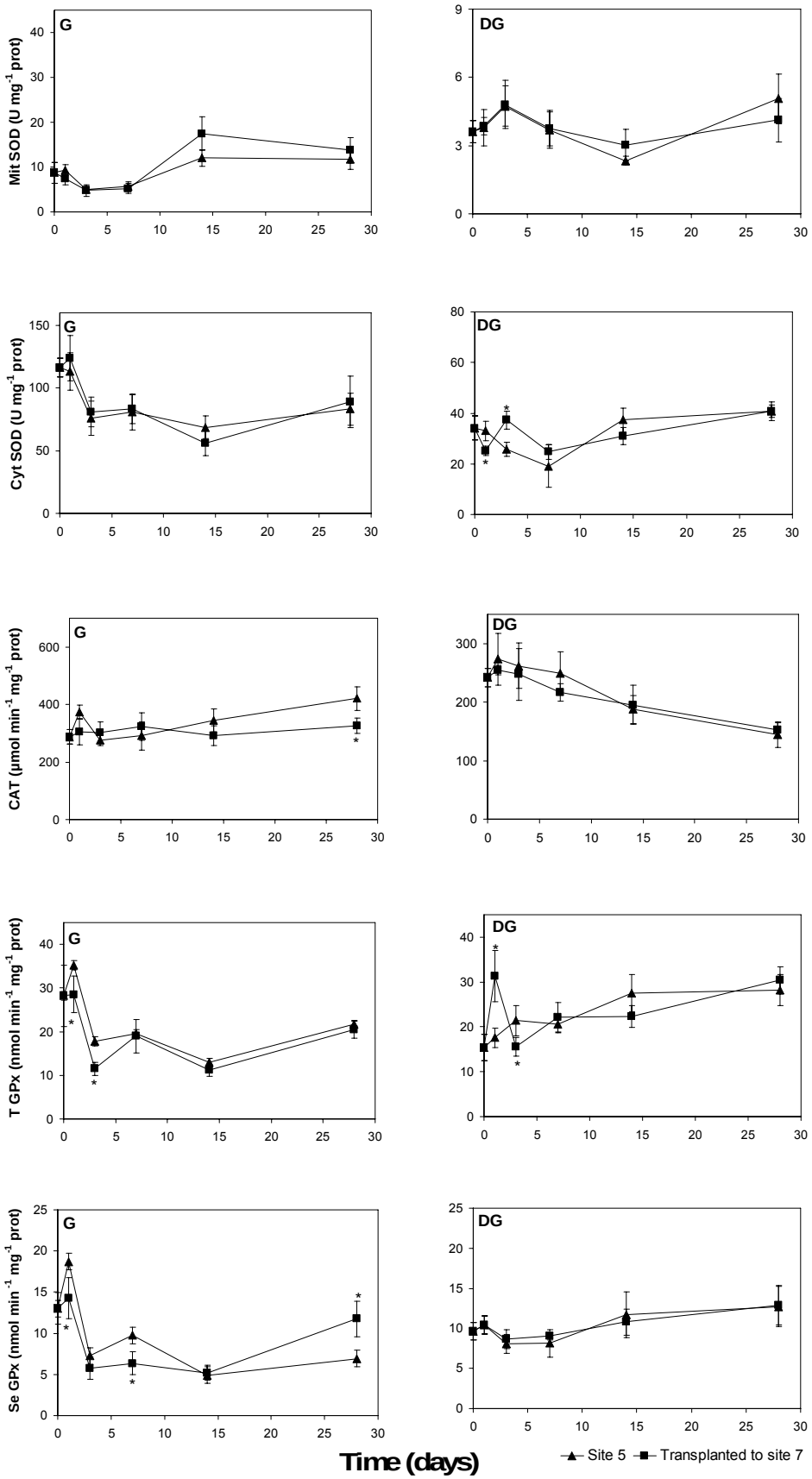


Figure 5.7 – Antioxidant enzymes activity (mean ± SD) in the gills (G) and digestive gland (DG), of clams from site 5 and transplanted to site 7. Significant differences between activity in clams from both sites are marked with * (p<0.05).

In this tissue, a linear decrease in Mit SOD activity was observed until day 3 (Mit $SOD_{Transplanted}$ = 8.75 – 1.34 t; r = -0.725; n = 21; p<0.05). Mit SOD activity significantly increased afterwards and by day 28 it was significantly higher than at the beginning of the experiment (p<0.05).

In the digestive gland, Mit SOD activity was significantly lower than in the gills (around 3 fold) (p<0.05) but not significantly different between sites. The activity of this enzyme showed some variability throughout the experiment: it significantly increased until day 3 and decreased until day 14 when it was significantly lower than at the beginning of the experiment. Between days 14 and 28, Mit SOD activity increased again reaching higher levels than at day 0 (p<0.05).

Like Mit SOD, Cyt SOD activity was approximately 2 fold higher in the gills than in the digestive gland (p<0.05). In the gills, the variation of Cyt SOD activity was similar in clams from both sites (p>0.05): it significantly decreased until day 15, remaining lower than at day 0 until the end of the experiment (p<0.05). Oppositely to the gills, the activity of Cyt SOD in the digestive gland changed differently between clam groups. In clams from site 5, Cyt SOD activity in the digestive gland, was significantly depressed until day 7 (Cyt $SOD_{Site\ 5}$ (U mg^{-1} prot) = 34.04 – 2.21 t (days); r = -0.762; n = 31; p<0.05), it significantly increased afterwards and, by the end of the experiment (day 28), it was significantly higher than at the beginning (p<0.05). In clams transplanted to site 7, Cyt SOD was significantly inhibited in the first day of the experiment and the activity was significantly lower than in clams from site 5 (p<0.05). However, this inhibition was transient and by day 3, Cyt SOD was induced and its activity was significantly higher than in clams from site 5. Afterwards Cyt SOD was inhibited again and induced linearly (Cyt $SOD_{Transplant}$ (U mg^{-1} prot) = 19.89 + 0.75 t (days); r = 0.902; n = 24; p<0.05), returning to the initial level by the end of the transplant experiment (day 28).

Similarly to Mit SOD, the variation of CAT activity in the gills was the opposite to that of the digestive gland. In the gills, CAT activity significantly increased in clams from site 5 at day 1. However, by day 3, CAT activity returned to the initial level. Afterwards, a linear increase

was observed until the end of the experiment ($CAT_{Site\ 5}$ (μmol min^{-1} mg^{-1} prot) = 256 + 5.93 t (days); r = 0.846; n = 15; p<0.05). In clams transplanted from site 5 to site 7, CAT activity remained unchanged during the experiment (p>0.05). Significant differences in CAT activity between clam groups were only observed at day 28 (lower in transplanted clams). In the digestive gland, CAT activity linearly decreased in clams from both sites with similar trends ($CAT_{Site\ 5}$ (μmol min^{-1} mg^{-1} prot) = 265 – 4.40 t (days); r = -0.817; n = 23; p<0.05 and $CAT_{Transplanted}$ (μmol min^{-1} mg^{-1} prot) = 249 – 3.54 t (days); r = -0.832; n = 23; p<0.05) and no difference exists in CAT activity between sites.

Oppositely to SOD and CAT activities, the levels of Total and Se GPx activities were similar in the gills and digestive gland (p>0.05) but the variation with time was different between tissues (p<0.05). In the gills, T GPx activity variation was similar between clam groups (significant differences were only observed at days 1 and 3). T GPx activity significantly increased at day 1, followed by a sharp decrease (at day 3) to levels significant lower than at day 0. Afterwards, the activity fluctuated until the end of the experiment, reaching levels significantly lower than at the beginning (p<0.05). In the digestive gland, T GPx activity increased linearly in clams from site 5 ($T\ GPx_{Site\ 5}$ (nmol min^{-1} mg^{-1} prot) = 17.97 + 0.43 t (days); r = 0.762; n = 45; p<0.05). In clams transplanted from site 5 to 7, however, a significant induction of T GPx occurred on the first day, which was significantly higher than in clams from site 5. By day 3, T GPx activity decreased to the original levels. After that period, it increased continuously until the end of the experiment ($T\ GPx_{Transplanted}$ = 15.7 + 0.53 t; r = 0.853; n = 33; p<0.05) to levels significantly higher than that at the beginning.

Although significantly lower than T GPx, the activity of Se GPx activity was also similar in the gills and digestive gland of clams from both sites (p>0.05). In the gills, Se GPx was significantly induced in clams from site 5 at day 1 and the activity decreased from day 3 until the end of the experiment to levels significantly lower than at day 0. In clams transplanted to site 7

Se GPx activity in the gills was significantly lower at days 1 and 7 than in clams from site 5 and reached significantly higher activities at the end of the experiment. By that time, the activity of Se GPx was not significantly different from that at day 0. In the digestive gland, Se GPx activity was similar in clams from both sites ($p>0.05$). Se GPx activity remained unchanged until day 7 and increased afterwards in clams from site 5 (significantly higher levels than at day 0 were only reached by day 28 in clams from both sites). In clams transplanted to site 7, Se GPx activity varied linearly throughout the experiment ($Se\ GPx_{Transplanted}$ (nmol min^{-1} mg^{-1} prot) = 9.14 + 0.12 t (days); $r = 0.613$; n = 40; $p<0.05$).

Figure 5.8 presents the variation of benzo[a]pyrene hydroxylase (BPH) and glutathione S-transferase (GST) activities along with the lipid peroxidation products (MDA and 4-HNE) in clams from site 5 and those transplanted to site 7 (see also Table A13 in Annexe).

No BPH activity was detected in the gills of *R. decussatus*. Oppositely to the antioxidant enzymes, the variation of BPH activity with time was different between clam groups. While in clams from site 5 BPH activity in the digestive gland linearly decreased throughout the experiment ($BPH_{Site\ 5}$ (f.u. min^{-1} mg^{-1} prot) = 0.85 – 0.03 t (days); $r = -0.781$; n = 18; $p<0.05$), in clams transplanted to site 7, BPH significantly increased at day 1 and decreased exponentially afterwards ($BPH_{Transplanted}$ (f.u. min^{-1} mg^{-1} prot) = $1.65e^{-0.09t\ (days)}$; $r = -0.689$; n = 18; $p<0.05$). By the end of the experiment BPH activity was significantly lower than at the beginning in clams from both sites ($p<0.05$).

Similarly to SOD, GST activity was significantly higher in the gills than in the digestive gland ($p<0.05$). In the gills, the variation of GST activity was similar in clams from both sites and presented a similar trend to that of T and Se GPx (Figure 5.7). GST was significantly induced in the two clam groups in the first day of the experiment, followed by a decrease to the initial level by day 3. Afterwards, GST activity increased linearly with time in clams from site 5 ($GST_{Site\ 5}$ (nmol min^{-1} mg^{-1} prot) = 753 + 31.4 t (days); $r = 0.813$; n = 26; $p<0.05$), while in those

transplanted to site 7, it remained unchanged until day 14, increasing then until the end of the experiment to levels significantly higher than that at the beginning in both clam groups.

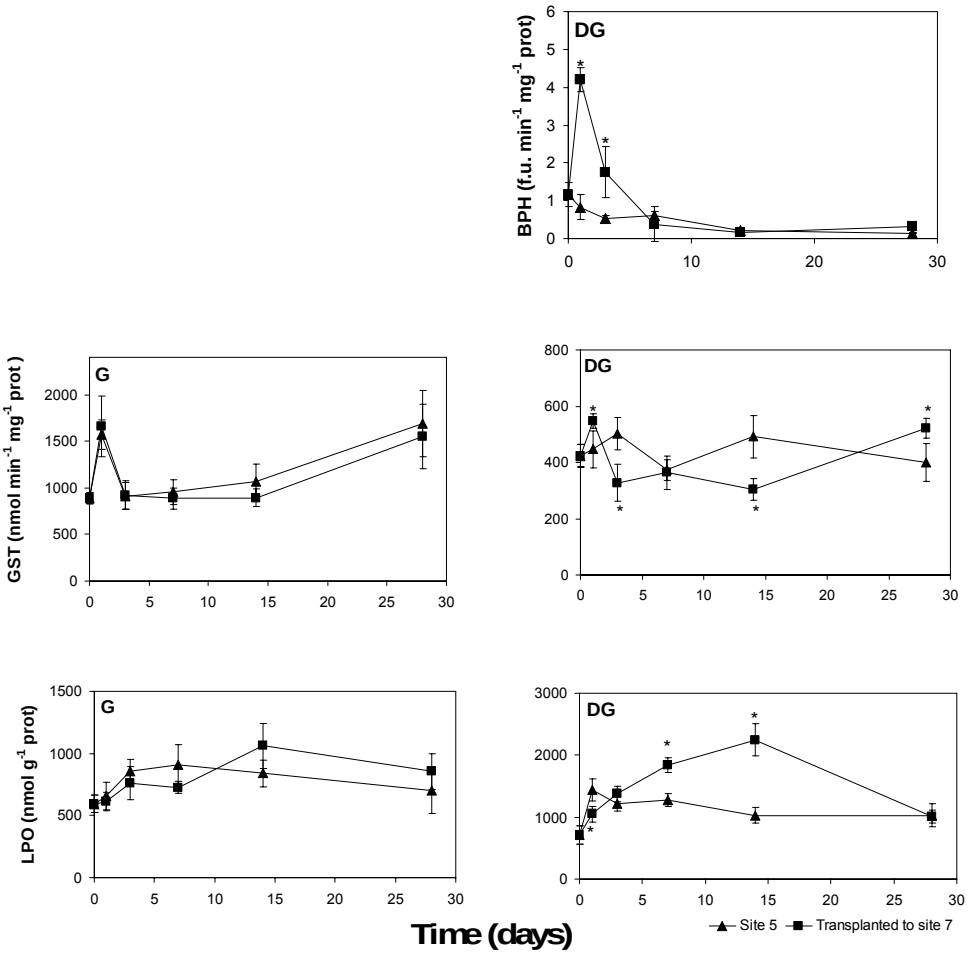


Figure 5.8 – BPH and GST activities and LPO (mean ± SD) in the gills (G) and digestive gland (DG), of clams from site 5 and transplanted to site 7. Significant differences between activity in clams from both sites are marked with * (p<0.05).

In the digestive gland of clams from site 5, GST activity increased linearly ($GST_{Site\ 5}$ (nmol min^{-1} mg^{-1} prot) = 423 + 26.3 t (days); r = 0.562; n = 22; p<0.05) in the first three days of the experiment. However, such increase was transient and afterwards GST activity decreased to its initial levels remaining unchanged until the end of the experiment. In clams transplanted to site 7, and similarly to BPH, GST activity increased on the first day, to significantly higher levels than in clams from site 5. Then, GST activity decreased (day 3) and was significantly different when compared with levels in clams from site 5. From day 3 to 14, GST levels

remained unchanged and were significantly lower than at day 0 although by the end of the experiment, GST was significantly induced again ($p<0.05$).

Oppositely to antioxidant enzyme activities, LPO was significantly higher (2-fold) in the digestive gland than in the gills ($p<0.05$). In the gills, LPO levels were similar in clams from both sites ($p>0.05$). In clams from site 5, LPO increased significantly at day 3, remaining higher than at day 0 until day 14. LPO then decreased until day 28 to levels similar to those at day 0. In clams transplanted to site 7, though, LPO increased linearly with time until day 14 ($LPO_{Transplanted}$ (nmol g^{-1} prot) = 597 + 30.5 t (days); r = 0.805; n = 17; $p<0.05$) and unchanged afterwards, levels remained higher than at day 0 until the end of the experiment. In the digestive gland, LPO varied differently between sites. In clams from site 5, LPO significantly increased at day 1 and remained unchanged and significantly higher than at day 0, until the end of the experiment. In clams transplanted to site 7, LPO increased linearly until day 14 ($LPO_{Transplanted}$ (nmol g^{-1} prot) = 930 + 103 t (days); r = 0.929; n = 20; $p<0.05$), after what LPO decreased to levels similar to those of clams at site 5.

B. Backtransplant Experiment

Figure 5.9 shows the variation of antioxidant enzymes of clams in the backtransplant experiment. In this experiment, antioxidant enzymes presented less variability with time and between tissues compared with the transplant experiment (see also Table A14 in Annexe).

Mit SOD activity in the gills remained unchanged in both clam groups until day 35. After that period and until the end of the experiment, Mit SOD activity in clams from site 5 increased linearly (Mit $SOD_{Site\ 5}$ (U mg^{-1} prot) = -26.8 + 0.98 t; r = 0.815; n = 28; $p<0.05$) while in those backtransplanted from site 7 to site 5, it remained unchanged ($p>0.05$).

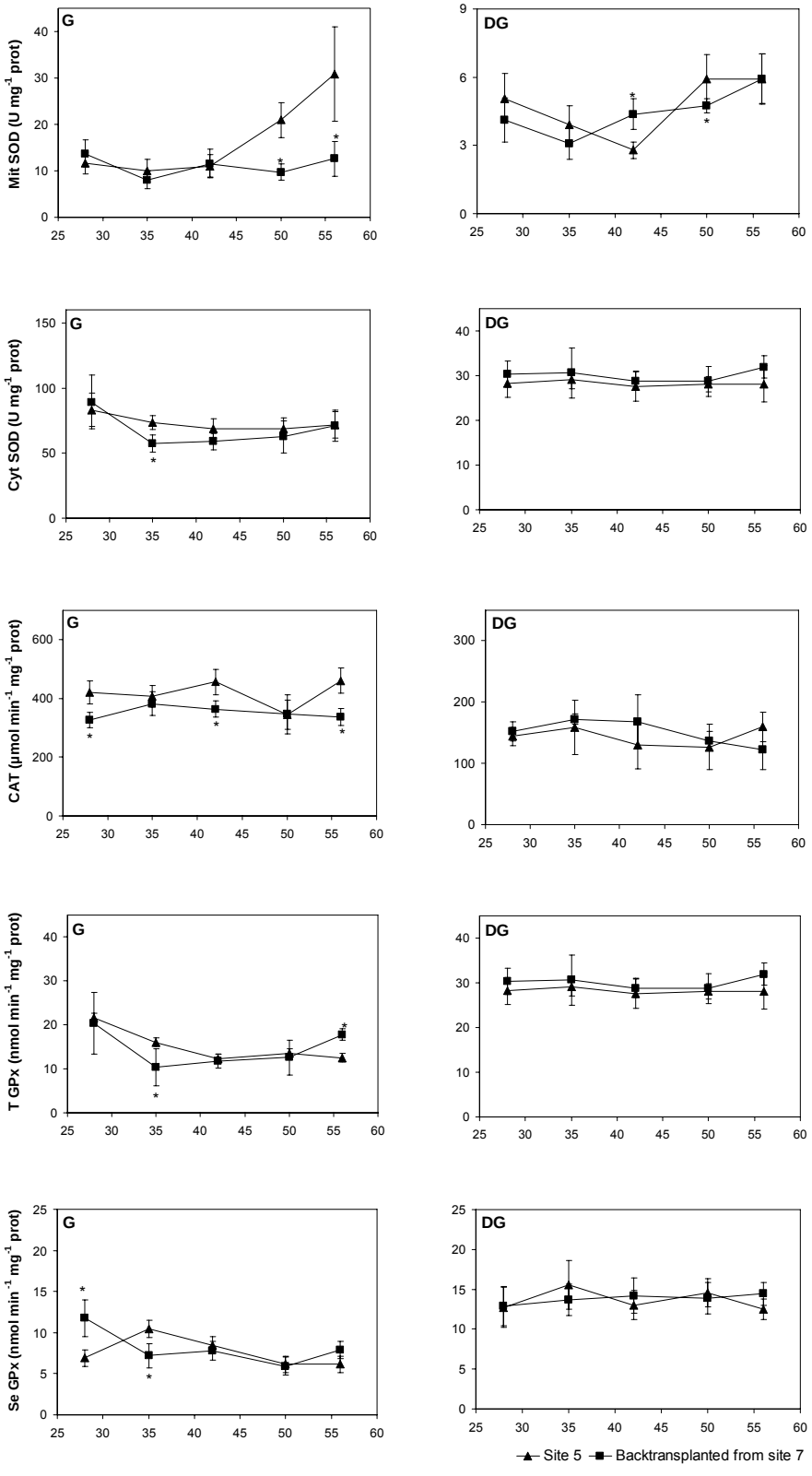


Figure 5.9 – Antioxidant enzymes activity (mean ± SD) in the gills (G) and digestive gland (DG), of clams from site 5 and backtransplanted from site 7 to site 5. Significant differences between activity in clams from both sites are marked with * (p<0.05).

In the digestive gland of clams from site 5, Mit SOD activity continued to show the same variability as in the first part of the experiment (Figure 5.7). Mit SOD activity significantly decreased until day 42 (Mit $SOD_{Site\ 5}$ (U mg^{-1} prot) = 9.59 – 0.16 t (days) ; r = -0.761; n = 23; p<0.05) and increased again until day 50, remaining unchanged until the end of the experiment (day 56). In clams backtransplanted from site 7 to site 5, a similar pattern was observed a week in advance. In the digestive gland, Mit SOD activity increased linearly between day 35 and day 56 (Mit $SOD_{Backtransplanted}$ (U mg^{-1} prot) = -1.01 + 0.12 t (days); r = 0.793; n = 25; p<0.05).

Cyt SOD activity in the gills and digestive gland was similar between clam groups. Significant differences were found only at day 35 (gills). In the gills of clams from site 5, although Cyt SOD activity significantly decreased until day 50 (p<0.05), it returned to the initial value (day 28) by the end of the experiment (day 56). In the gills of clams backtransplanted from site 7 to site 5, Cyt SOD activity significantly decreased at day 35, and by the end of the experiment, no differences were observed between Cyt SOD activity in clams from both sites (p>0.05). In the digestive gland, Cyt SOD remained unchanged in both clam groups.

CAT activity, though presenting some variability during the course of the backtransplant experiment, was not significantly modified in the gills or digestive gland of clams from site 5. In the gills of backtransplanted clams, however, CAT activity increased during the first week of the experiment, decreasing afterwards. By the end of the experiment, CAT activity was similar to that of day 28 (p>0.05). In the digestive gland, there was no variation in CAT activity in clams from site 5 (p>0.05). In clams backtransplanted from site 7 to site 5, CAT activity remained unchanged until day 42, after what it decreased linearly until the end of the experiment ($CAT_{Backtransplanted}$ (μmol min^{-1} mg^{-1} prot) = 307 – 3.33 t (days); r = -0.766; n = 11; p<0.05).

Oppositely to what was observed in the transplant experiment, T GPx and Se GPx activities, in the backtransplant experiment, were significantly lower in the gills than in the digestive gland (p<0.05). In the gills of clams from site 5, T GPx activity decreased

logarithmically throughout the experiment (T $GPx_{Site\ 5}$ (nmol min^{-1} mg^{-1} prot) = 60.00 – 12.07 ln t (days); r = 0.728; n = 39; p<0.05). In backtransplanted clams, however, after a decrease in T GPx activity in the first week of the experiment, to levels significantly lower than in clams from site 5, the activity remained unchanged until day 50, and it increased again reaching levels significantly higher than in clams from site 5. In the digestive gland, T GPx activity remained unchanged during the experiment and was similar in both clam groups (p>0.05).

Similarly to T GPx, Se GPx activity variation with time was similar in clams from both groups in the digestive gland but not in the gills. In the gills of clams remaining at site 5, Se GPx activity significantly increased during the first week of the experiment, decreasing afterwards (Se $GPx_{Site\ 5}$ (nmol min^{-1} mg^{-1} prot) = 17.47 – 0.21 t (days); r = -0.786; n = 28; p<0.05). In backtransplanted clams, however, Se GPx activity significantly decreased in the first week, remaining unchanged and lower than at day 28 during the rest of the experiment. In the digestive gland Se GPx remained unchanged during the experiment and similar between clam groups.

Figure 5.10 presents the variation with time of BPH and GST activities and LPO products in the gills and digestive gland of clams during the backtransplant experiment (see also Table A15 in Annexe).

Compared to the initial part of the experiment and similarly to the antioxidant enzymes, the variation of BPH, GST and LPO with time was less pronounced during the backtransplant experiment.

BPH activity remained unchanged and similar in both clam groups until day 42, increasing significantly only at day 50. Such increase was however significantly higher in backtransplanted clams than in clams remaining at site 5. By the end of the experiment, although levels had decreased in backtransplanted clams, BPH activity was still significantly higher than in clams from site 5.

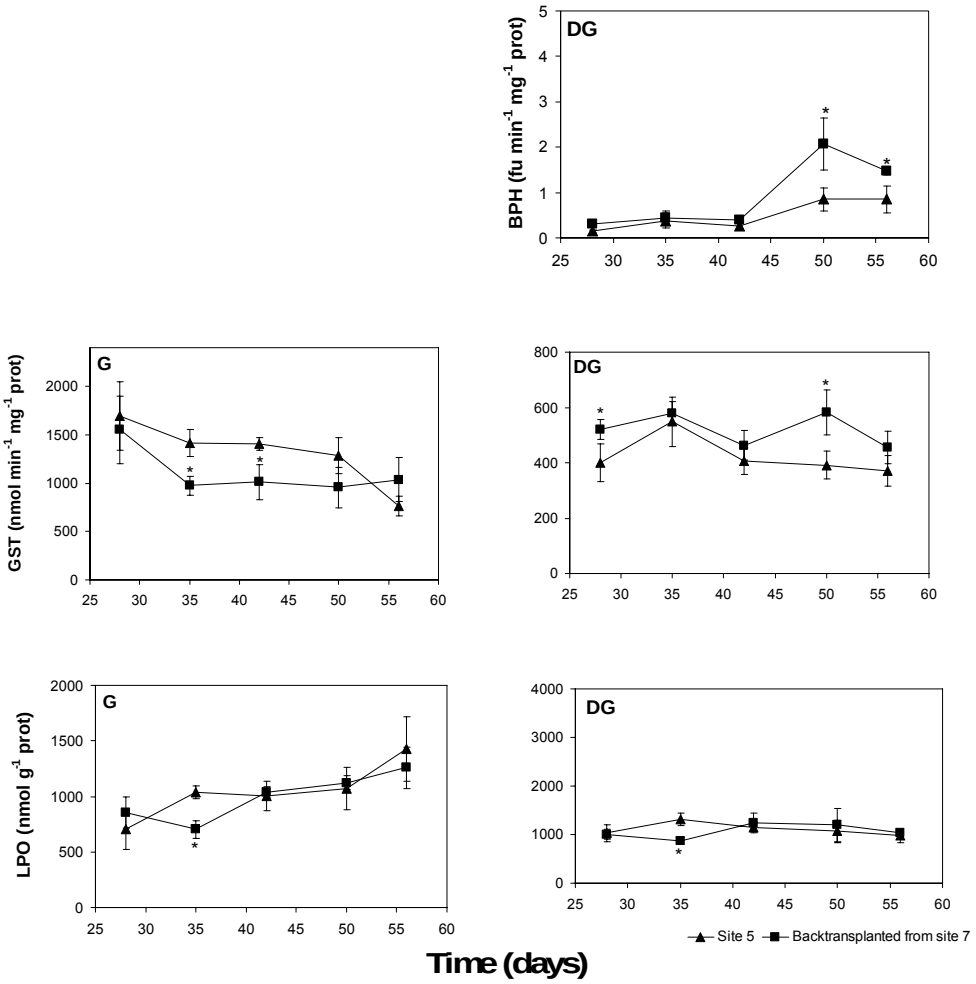


Figure 5.10 – BPH and GST activities and LPO (mean ± SD) in the gills (G) and digestive gland (DG), of clams from site 5 and backtransplanted from site 7 to site 5. Significant differences between activity in clams from both sites are marked with * (p<0.05).

GST activity followed the same pattern in both clam groups in the gills and digestive gland. Similarly to the initial part of the experiment, GST activity was approximately 3-fold higher in the gills than in the digestive gland (p<0.05). In the gills, a general decrease was observed, which was linear in clams from site 5 ($GST_{Site\ 5}$ (nmol min^{-1} mg^{-1} prot) = 2429 – 26.29 t (days); r = -0.758; n = 36; p<0.05). In backtransplanted clams, GST activity significantly decreased during the first week and remained unchanged until the end of the experiment (p>0.05).

In the digestive gland GST activity did not vary significantly throughout the experiment (p>0.05). However, differently from the gills, GST activity was higher in backtransplanted than in clams from site 5 at days 28 and 50.

The concentration of LPO products varied similarly in clams from both sites, although differently between tissues. In the gills, LPO increased linearly during the experiment ($LPO_{Site\ 5}$ (nmol g^{-1} prot) = 186 + 20.71 t (days); r = 0.736; n = 18; p<0.05 and $LPO_{Backtransplanted}$ (nmol g^{-1} prot) = 267 + 17.26 t (days); r = 0.786; n = 20; p<0.05). In the digestive gland, LPO levels remained unchanged in both clam groups but were significantly lower in the backtransplanted clams at day 35 in both tissues.

5.3.4. Relationship between PAHs concentrations and biochemical parameters

The relationship between PAHs accumulated in clam whole soft tissues and the biochemical parameters either in the gills or in the digestive gland of *R. decussatus* was assessed with Spearman by Ranks Correlations (for non-parametric data) and PLS analysis were performed separately for the transplant and backtransplant experiments.

A. Transplant Experiment

Figure 5.11 presents the weightings plot for the transplant experiment. It is obvious from this plot that there is a group of enzymes which are positively related to acenaphthene (Ac) and benzo[*a*]anthracene (BaA). These are: Cyt SOD, T GPx, Se GPx and GST in the gills and Mit SOD, BPH and GST in the digestive gland (circle A in Figure 5.11). However, only the correlation between BaA and GST in the gills is significant (p<0.05). Apart from Ac and BaA, most of the 3 and 4 ring PAHs appear to have an antagonistic effect on antioxidant enzymes since they are located on opposite sides of this plot. Spearman Rank Order Correlations were

significant between fluorene (F) and BPH (-0.673), CAT DG (-0.664) and Se GPx Gills (-0.745); and between phenanthrene (P) and anthracene (A) and Cyt SOD DG (-0.645 and -0.718, respectively).

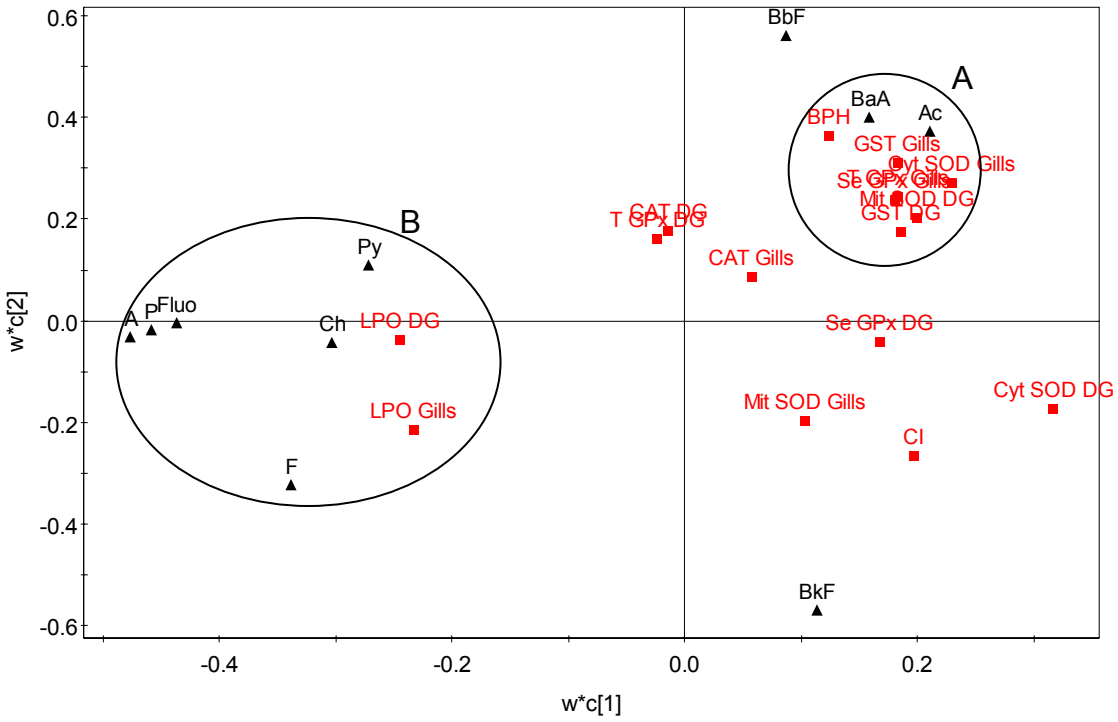


Figure 5.11 – PLS weights plot of the individual PAHs as concentrations (X-variables) and biochemical parameters (Y-variable) of clams from both sites during the transplant experiment (11 *observations*; 26 *variables*). The first component explained 40.2% and the second component 24.7% of the variance in the data (both significant).

LPO in both tissues is directly related to most 3 and 4 ring PAHs (circle B in Figure 5.11). In fact, positive Spearman correlations exist between LPO and P and A concentrations (0.609 and 0.782 respectively). LPO is also located opposite to most antioxidant enzymes, meaning that it was highest when antioxidant enzymes activity was lowest.

In a different approach, Figure 5.12 presents the amount of variance within the biochemical parameter data explained and predicted by the PAH data.

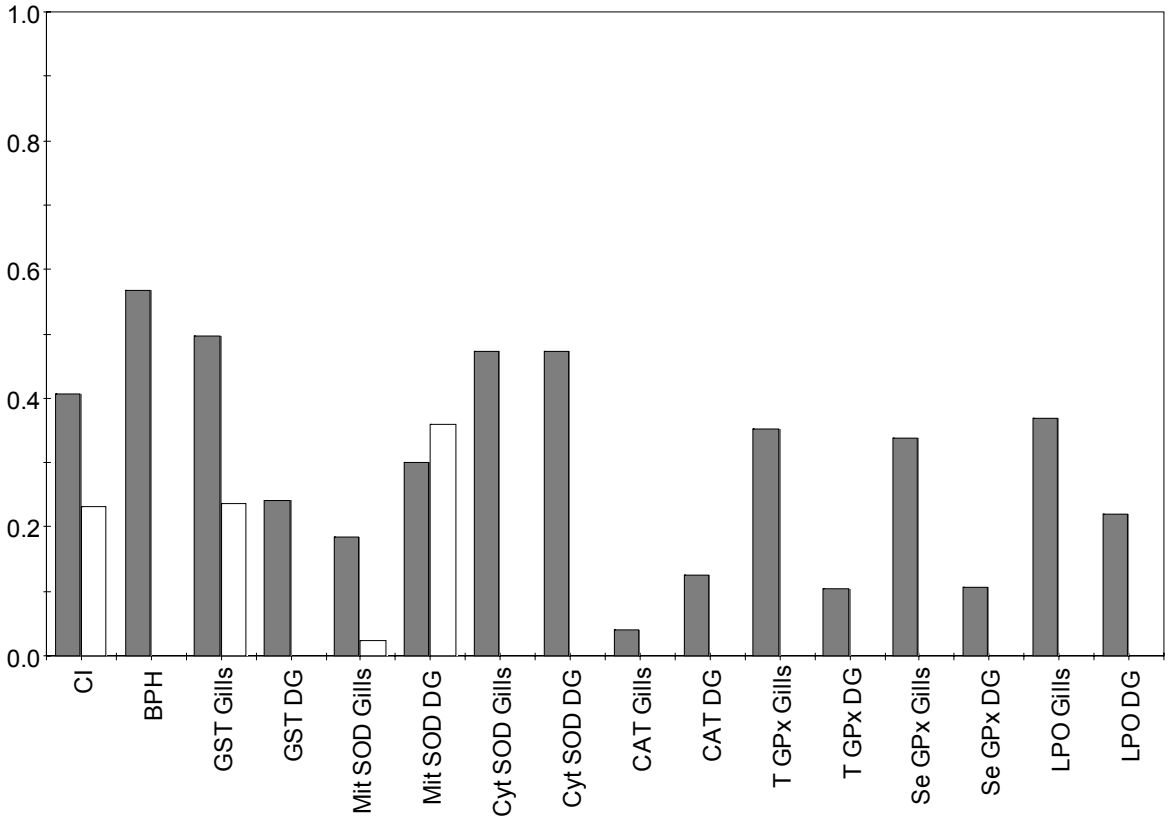


Figure 5.12 – Partial Least Squares analysis using the PAH concentrations as X-block. Bars show the amount of variance within the biochemical parameters data explained (dark grey) and predicted (open bars) by the PAHs in the transplant experiment.

Generally, PAH concentrations explain only small amounts of the variance in the biochemical parameters (less than 60%). The highest explained variances were for the CI (Condition Index), GST, Cyt SOD, T GPx, Se GPx and LPO in the gills and BPH and Cyt SOD in the digestive gland, all with explained variances higher than 35%. This means that in the transplant experiment some of the variation in these enzyme activities could be related to PAH concentrations in the clam whole soft tissues. The predicted variances, however, were zero for most these enzymes, except for CI, GST Gills and Mit SOD DG. The most influent PAHs in this analysis were Ac, BaA, P, A and Fluo.

B. Backtransplant Experiment

Figure 5.13 shows the PLS weights plot for the backtransplant experiment. Comparing with the PLS weights plot from the transplant experiment, the variables position in this plot varied considerably.

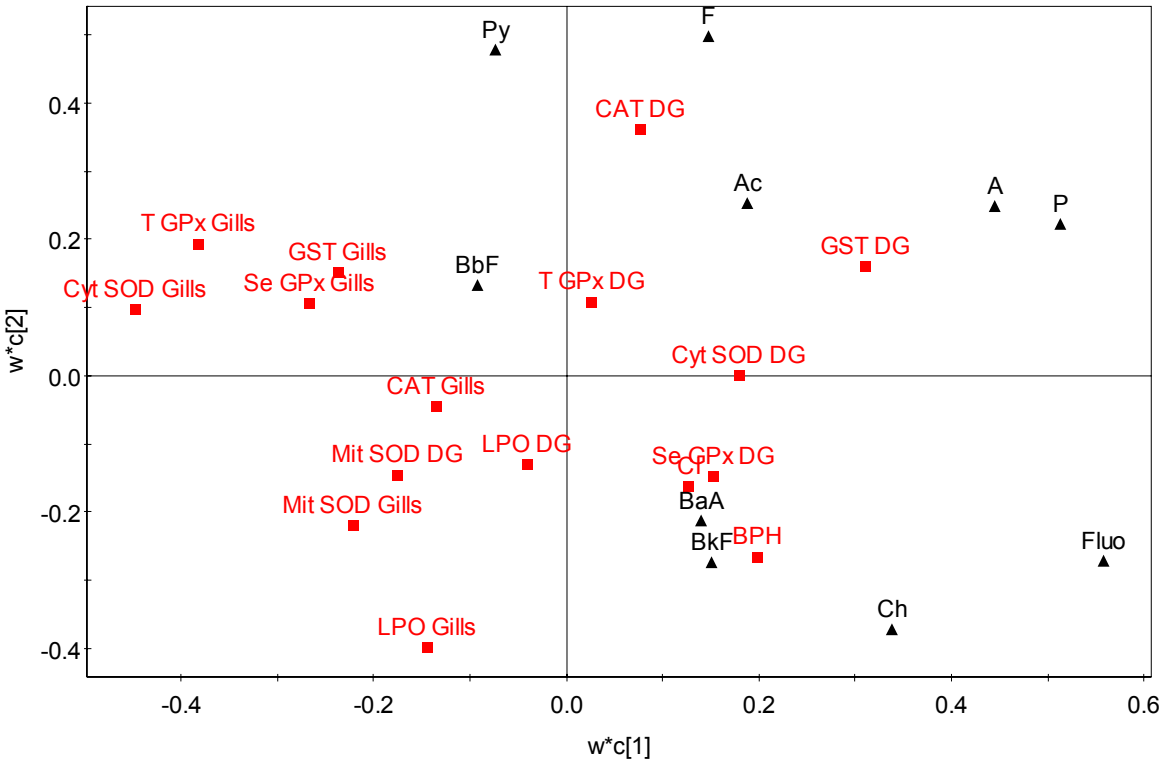


Figure 5.13 – PLS weights plot of the individual PAHs as concentrations (X-variables) and biochemical parameters (Y-variable) of clams from both groups during the backtransplant experiment (10 *observations*; 26 *variables*). The first component explained 34.4% and the second component 32.5% of the variance in the data (both significant).

The biochemical parameters are now separated by tissue: gills on the left side of the plot and digestive gland on the right with the exception of Mit SOD and LPO in the digestive gland which are very near the same parameters in the gills. The most relevant association is the inverse relationship between Cyt SOD and T GPx activity in the gills and most of the 3 and 4 ring PAHs, especially P, A, Fluo and Ch. Cyt SOD Gills was negatively correlated with A (-0.636), Fluo (-

0.855) and Ch (-0.709) and T GPx Gills with A (-0.673) and Fluo (-0.661). Spearman Correlations were negative between BbF and BPH (-0.697) and between Py and LPO Gills (-0.661) and positive between P and GST DG (0.745) and between A and CAT DG (0.697).

Figure 5.14 presents the amount of variance within the biochemical parameter data explained and predicted by the PAH data in the backtransplant experiment.

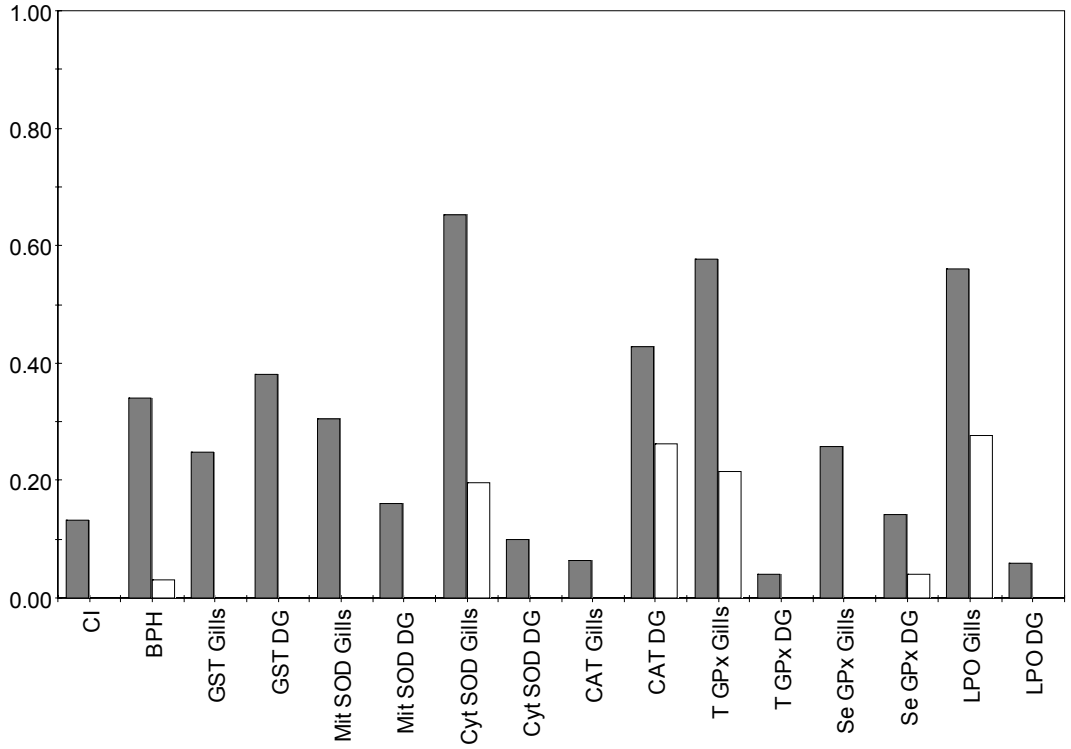


Figure 5.14 – Partial Least Squares analysis using the PAH concentrations as X-block. Bars show the amount of variance within the biochemical parameters data explained (dark grey) and predicted (open bars) by the PAHs in the backtransplant experiment.

Compared with the transplant experiment, the amount of explained variance increased in this experiment for some variables though it decreased for others. However, on average the amount of variance explained was similar in the two experiments. The most relevant increases were for Cyt SOD, T GPx and LPO in the gills and CAT in the digestive gland. Explained variances higher than 35% were observed only for GST, Cyt SOD, T GPx and LPO in the gills and for BPH and CAT in the digestive gland. The amount of predicted variance is low, in this

experiment. The CI, GST Gills and Mit SOD DG may no longer be predicted by the individual PAHs, however, the more predictable variables are now those where the amount of explained variance increased as well. The most influent PAHs in this analysis were P, A and Fluo.

Combining the results of the two experiments, PAHs appear to exert the highest influence on GST, Cyt SOD, T GPx and LPO in the gills and on BPH in the digestive gland and Ac, BaA, P, A and Fluo appear to be the most influent PAHs.

5.4. DISCUSSION

The main goal of this experiment was to analyse PAHs accumulation and its effects in real environmental conditions. For that purpose, clams were transplanted from site 5 (Marim) to site 7 (Tavira), which previously revealed significantly different PAH levels in sediment and clam tissues in January – May (Chapters 2 and 3).

Despite the fluctuations of the condition index (CI) of clams from both sites (Table 5.1), clams health conditions did not change throughout the experiment and the CI levels were similar to those reported by Leite *et al.* (2004) for the same species.

5.4.1. Transplant Experiment

An increase in aromatic hydrocarbons concentration occurred on the first day, which was significantly higher in clams transplanted to site 7 (Figure 5.1). Such an increase reflects the input of PAHs to this coastal lagoon and the intense rainfall (15.2 mm) occurred on the first day of this experiment (http://snirh.inag.pt). Rainfall may contribute to the input of PAHs to the aquatic environment in several ways, including wet precipitation from the atmosphere (Golomb *et al.*, 2001) and urban runoff (Dickhut *et al.*, 2000).

The accumulation of PAHs in bivalve tissues is the result of the balance between the uptake and elimination rates (Neff, 2002). Usually an inverse relationship between uptake rate of PAHs and its K_{ow} exists (Sericano *et al.*, 1996; Neff, 2002). Most filter and suspension feeder bivalves accumulate organic contaminants directly from the water-phase. Thus, the more soluble PAHs (with lower K_{ow}) are more readily absorbed by the organisms than the less soluble PAHs which are generally adsorbed to particles.

The increase in tPAH concentrations in clam tissues on the first day of the experiment (figure 5.1) was mostly due to the accumulation of the 2+3 ring PAHs (Figure 5.2) and particularly Ac, BaA and Ch in clams from site 5 while in transplanted clams besides Ac and BaA, Py was also preferentially accumulated (Figure 5.3). The accumulation of Ac in clams from both sites reflects not only its higher solubility in water but also the fact that water is the preferential uptake pathway by *R. decussatus* of organic contaminants rather than sediments or food as detected for TBT uptake in the same species (Coelho *et al.*, 2002a; 2002b). Although less soluble than the 3 aromatic ring PAHs, BaA accumulation in clams from site 5 could be due to the presence of higher concentrations of this PAH at this site at that time pointing to the existence of different PAH sources to both sites 5 and 7.

PAH levels and signatures were distinct between sites. Site 7 is located on the proximity of the River Gilão which is the only relevant freshwater input to the Ria Formosa. It is also impacted by the discharges from a wastewater treatment plant which serves a population of around 30 000 habitants, and is under the influence of a recreational and fishing harbour (see section 3.4 for further detail). On the contrary, site 5 is located at an inner part of the Ria Formosa, away from anthropogenic influence. This site may however receive PAHs due to run-off and wet precipitation.

Following the rapid increase on the first day of the experiment, PAH concentrations significantly decreased, mostly due to the elimination of Ac (Figure 5.3). Similarly to uptake, an

inverse relationship exists between elimination rates of PAHs and its K_{ow} (Sericano *et al.*, 1996; Neff, 2002). That is, the more soluble PAHs are eliminated or biotransformed more easily than the more hydrophobic ones (Neff, 2002). tPAH concentration (Figure 5.1), 2+3 and 4 ring PAHs (Figure 5.2), mainly Ac, BaA and Ch decreased more rapidly in clams from the transplant site (Figure 5.3) reflecting the easy uptake and elimination of the compounds by these organisms at this site. tPAH concentrations in clams from site 5 behaved differently (Figure 5.1). The 2+3 ring PAHs were continuously accumulated during the whole experiment while 4 ring PAHs also increased but with some variability. By the end of the experiment PAH levels were higher than at the beginning of the experiment and than from the transplant site (Figures 5.1 and 5.2). Among the individual PAHs, Ac, F, Py, BaA and BbF were the most accumulated in the clams at this site (Figure 5.3).

In what concerns the biomarkers, the activities of several enzymes (GST, SOD and CAT) were significantly higher in the gills of *R. decussatus* while for T and Se GPx, levels were similar and LPO the opposite (Figures 5.7 and 5.8). These results confirm those reported for the same species in the Ria Formosa (Geret *et al.*, 2003) and may represent a first line of defence against tissue exposure or damage especially for the more soluble contaminants (particularly Ac) mainly absorbed through the gills (Cossu *et al.*, 2000; Cheung *et al.*, 2001). On the contrary, BPH activity was only detected in the digestive gland (Figure 5.8) indicating that this organ is probably the main responsible for PAHs metabolism inducing also higher LPO levels (Peters *et al.*, 1999).

Most of the enzymes (Figures 5.7 and 5.8) were similar between sites despite the differences in PAH levels (Figure 5.1). It is possible that the differences in PAH concentrations between sites were not enough to induce distinct levels of biomarker responses. There is no information on the PAH concentration required to induce these enzymes in *R. decussatus*.

Therefore, in future transplant studies involving this species, more contrasting sites should be chosen in order to observe spatial differences in biomarker responses.

Despite the lack of differences between sites, most of the enzymes which changed during the experiment could be related to the variation of individual PAH concentrations. Individual PAHs induce different biomarker responses: Ac and BaA are directly related to Cyt SOD, T and Se GPX and GST in the gills and Mit SOD, BPH and GST in the digestive gland. Most of the 3 and 4 aromatic ring PAHs (F, A, P, Fluo, Py and Ch), however, were directly related to LPO in both gills and digestive gland and negatively related to Cyt SOD in the gills.

Biomarker responses in the gills indicate the presence of superoxide anion responsible for Cyt SOD induction (Lemaire & Livingstone, 1997). Cyt SOD activity in this bivalve species is mainly the result of Cu/Zn-SOD activity (Geret *et al.*, 2002a). Cu/Zn-SOD has at least three distinct isoforms in the blue mussel *M. edulis*, which contribute to the cytosolic activity of this enzyme (Manduzio *et al.*, 2003). However, only one of them was induced in the presence of xenobiotics while total SOD activity remained unaltered (Manduzio *et al.*, 2003; Manduzio *et al.*, 2004). Cu/Zn-SOD cDNA has already been isolated in *R. decussatus* (Geret *et al.*, 2004) and similar isoforms as in *M. edulis* exist (Geret, F., unpublished results), however, their full sequence needs to be confirmed (Bebianno *et al.*, 2004). The production of H_2O_2 by SOD in the gills was not enough to induce CAT activity (Kinnula *et al.*, 1992). Instead, Se GPx was induced in the gills suggesting that this enzyme is the main responsible for the hydrogen peroxide detoxification instead of CAT (Matés, 2000). T GPx induction also indicates that PAHs and/or other organic compounds metabolisation are responsible for the production of membrane lipid peroxides (Van der Oost *et al.*, 2003). Since no activity of BPH was detected in the gills, GST is probably acting as a peroxidase rather than as a Phase II enzyme (Varanasi *et al.*, 1989; Sheehan *et al.*, 2001). This would explain the similar variation of GST and T and Se GPx activities (Figures 5.7; 5.8 and 5.11). Similar inductions in GST and T GPx activities were observed in

mussels *Perna viridis* transplanted to a PAHs contaminated site in Hong Kong though SOD and LPO were not affected by the transplant (Cheung *et al.*, 2001).

Oppositely to the gills, BPH in the digestive gland was the biomarker with the highest explained variance (60%; Figure 5.12). The increase of BPH activity is related to an increase of phase I biotransformation process leading to the formation of phenols or diols. The induction of BPH was also observed in the digestive gland of mussels *Mytilus edulis* transplanted to a PAH contaminated site in the Faroe Islands, North Sea (Solé *et al.*, 1998) and in clams *Mercenaria mercenaria* transplanted to PAH, PCB, DDT and metals contaminated sites in Tampa Bay, Florida (Nasci *et al.*, 1999). BPH was induced by Ac and BaA in the digestive gland of *R. decussatus* (Figure 5.11). The structure of BaA is similar to benzo[*a*]pyrene, containing several "k-regions" where epoxides may be formed, which by further metabolisation originate diols and phenols (Yang & Chiu, 1985; Yang, 1988; Varanasi *et al.*, 1989). Ac, however, does not present a "k-region", therefore its relationship with BPH remains unclear. The arrangement of the aromatic rings in the BaA molecule contains also a "bay-region" often correlated with carcinogenic properties (Carberry *et al.*, 1988; Penning, 1993). More studies on the PAH biotransformation mechanism are necessary in order to clarify this point. The involvement of BaA in redox cycling mechanisms and generation of reactive oxygen species has also been demonstrated in several mammalian studies (Penning, 1993; Fabiani *et al.*, 1999).

BPH is directly related to GST in the digestive gland which suggests an important role for GST in the biotransformation of PAHs in this tissue. In addition, the direct relationship between BPH and Mit SOD in the digestive gland (Figure 5.11) indicates the redox cycling of Phase I products (phenols and diols) (Penning, 1993; Lemaire & Livingstone, 1997; Fabiani *et al.*, 1999), leading to the production of radical oxygen species and inducing protective antioxidant enzymes (Dicker & Cederbaum, 1991; Di Giulio *et al.*, 1995; Van der Oost *et al.*, 2003).

Similarly to the previous results of the seasonal and spatial study (Chapter 4), Cyt SOD and CAT in the digestive gland were negatively related to F, A, P and Fluo. This negative relationship results from the coincident decrease in these enzyme activities and maximum concentrations of these PAHs which occurred at day 7. Though this suggests that Cyt SOD and CAT were inhibited in the presence of these PAHs, further studies are needed in order to confirm this hypothesis.

The behaviour of LPO was the opposite of all the antioxidant enzymes (Figure 5.11) stressing the protective role of antioxidant enzymes in the protection of cells against oxidative stress. In fact, the highest LPO levels occurred in the digestive gland at day 14 (figure 5.8) after the decrease of most antioxidant enzyme activities, particularly SOD and CAT (Figure 5.7). Similarly, LPO products in mussels *P. viridis* transplanted to PAH contaminated sites in Hong Kong were significantly higher in the digestive gland than in the gills also associated with lower antioxidant enzyme defences in the digestive gland (Cheung *et al.*, 2001).

5.4.2. Backtransplant Experiment

During the backtransplant experiment, the variation of PAH concentrations was smaller than in the transplant experiment. However, despite the fact that both clam groups were now exposed to the same conditions, PAHs in the clams backtransplanted to the original site presented a different trend than those remaining at site 5. The initial PAH levels in clams at site 5 probably reflected the higher PAH concentrations at the beginning of the backtransplant (day 28) experiment at this site (Figure 5.4). Backtransplanted clams however took 7 days to reach this PAH level in its tissues. Despite presenting a similar variation in PAHs concentration with time after day 52, levels remained higher in backtransplanted clams than in clams at site 5 suggesting that clams did not have time to acclimate to the new conditions.

Similarly to the PAH concentrations, the variation of the biomarkers was also smaller than in the transplant experiment (Figures 5.9 and 5.10). Contrary to the transplant experiment, GPx activities were higher in the digestive gland (Figure 5.9) which is probably linked to the decrease in LPO in this tissue when compared to the gills (Figure 5.10).

Analysing the biomarkers as a response to the PAHs variation during the experiment (Figure 5.13), enzyme activities in the gills were the opposite of those in the digestive gland. Such behaviour was the most relevant feature in the backtransplant experiment pointing once more to the different responses of these organs to PAHs contamination.

The positive relationships between Ac and BaA and biomarkers in the gills in the transplant experiment did not occur in the backtransplant but in this backtransplant experiment Ac and BaA concentrations were significantly lower (Figures 5.3 and 5.6). In the gills, most biomarkers were negatively related with PAHs. This negative relationship was more significant between Cyt SOD and T GPx and A, P Fluo and Ch (Figures 5.13 and 5.14). Similarly, Cyt SOD and T GPx activities decreased in the gills of mussels *Unio tumidus* transplanted to a PAHs and metals contaminated site (Cossu *et al.*, 1997). These results are, however, different from those reported for mussels *Perna viridis* transplanted between relatively uncontaminated and PAH contaminated sites in the Hong Kong area in which increases in T GPx activity along with increasing PAH concentrations were observed (Cheung *et al.*, 2001).

In the digestive gland, BPH, GST and CAT were the best explained by the variance within the PAHs data (Figure 5.14) and were directly related to Ac and BaA (Figure 5.13) as in the transplant experiment, confirming their role in PAHs metabolism as discussed above. This is also supported by mussels *M. galloprovincialis* transplanted to more contaminated sites in the northwest Mediterranean Sea in which direct relationships between BPH and GST activities and accumulated PAHs exist (Bodin *et al.*, 2004; De Luca-Abbott *et al.*, 2005). The relationship between CAT and some PAHs (Ac, F and Py) indicates that H_2O_2 in particular was produced

though no induction of other antioxidant enzymes occurred. Similar CAT induction after PAHs accumulation was observed in the digestive gland of mussels *Unio tumidus* transplanted to a PAHs and metals contaminated site (Cossu *et al.*, 1997).

In conclusion, the clam *R. decussatus* exposed to environmental PAH concentrations is capable of metabolising and eliminating these aromatic compounds in a relatively short period of time. In this process, ROS are produced which lead to the induction of protective antioxidant enzymes (Cyt SOD and T and Se GPx in the gills and Mit SOD and CAT in the digestive gland) and cause oxidative damage to membranes e.g. lipid peroxidation (particularly in the digestive gland). BPH and GST activities and LPO products in the digestive gland appear to be the most relevant indicators of PAHs metabolisation in *R. decussatus*. Antioxidant enzymes, however, are general stress biomarkers which are induced by several environmental contaminants (Cajaraville *et al.*, 2000). The variation in these stress enzymes may also be due to contaminants other than PAHs (PCBs, DDT, metals, etc.) that occur in the Ria Formosa as discussed in section 4.4. This should be taken into account in future studies.

6. GENERAL DISCUSSION AND CONCLUSIONS

The use of organisms as bioindicators of chemical contaminants has been developed to monitor environmental pollution in coastal and estuarine areas (Wade *et al.*, 1998; Goldberg & Bertine, 2000). The interest in using organisms to monitor pollution relies on the fact that fugacious contamination may often not be easily detected in water or recorded in the sediment while bivalves may accumulate and therefore "record" the contaminants concentrations (Baumard *et al.*, 1998b). Moreover, the comparison of sediment and bivalve tissue PAH fingerprints can provide information on the bioavailability of the various PAHs as well as on the fate of these compounds once absorbed by bivalves (bioaccumulation, metabolism, and so on) (Baumard *et al.*, 1998a). *R. decussatus* like many other molluscs accumulates PAHs (Solé *et al.*, 1994; Porte *et al.*, 1996; Porte *et al.*, 1998; Solé *et al.*, 2000) which makes their use as bioindicator organisms important from the human health perspective and in areas where mussels are unavailable.

In early studies, monitoring was mainly focused on the contaminant occurrence and/or on its concentration but these data do not provide any information on the effects that they have on organisms. To bypass these limitations, the integrated use of chemical contaminants along with biomarkers of exposure and/or of effects was proposed. This combination has proved an effective means of evaluating the impact of pollution in the aquatic environment (Porte *et al.*, 1991; Solé *et al.*, 1994; Livingstone *et al.*, 1995; Solé, 2000a). However, the complexity of the natural environment, where mixtures of pollutants interact among themselves and with biological systems, makes the adoption of a single biomarker meaningless.

Antioxidant enzymes exist in biological systems as a means to eliminate reactive oxygen species, which may be produced by organisms when exposed to organic contaminants (Livingstone, 2001). These enzymes activity, along with lipid peroxidation products, have been proposed as biomarkers of PAHs exposure and effect (Porte *et al.*, 1991; Solé *et al.*, 1994; Solé *et al.*, 1995; Livingstone, 2001). Nevertheless, the use of these biochemical parameters as

biomarkers in field studies is difficult due to the influence of abiotic (temperature, salinity, sunlight exposure,…) and biotic (food availability, reproduction and parasitism) factors (Livingstone *et al.*, 1990; Sleiderink *et al.*, 1995; Cotelle & Férard, 1999; Sheehan & Power, 1999). Hence, the characterisation of these variations is vital for the validation of the use of biomarkers.

The seasonal variation of PAHs in sediments and in clam tissues along with four antioxidant enzymes (superoxide dismutase, catalase, total glutathione peroxidases and Se dependent glutathione peroxidases) and lipid peroxidation products were studied in *R. decussatus*.

The seasonal and spatial variation of PAH concentrations in the sediments from several clam farms representative of the contamination to which the clams were subjected to (Chapter 2) revealed that in the Ria Formosa lagoon PAH concentrations were comparable to those from the central part of the Gulf of Trieste (Notar *et al.*, 2001), or from remote or slightly urbanised and leisure areas of the Mediterranean Sea (Baumard *et al.*, 1998b; Table 1.3; Chapter 1). However, PAH levels in the sediments from the Ria Formosa lagoon (Figure 2.5; Table A2) were slightly contaminated (tPAH <250 ng/g; Notar *et al.*, 2001) and lower than the ecotoxicological assessment criteria defined for surface sediments by the OSPAR Commission (OSPAR Commission, 2000). PAH concentrations and distribution pattern were mostly affected by seasonal rather than by spatial effects (Figures 2.9 and 2.10). That is, both PAH concentrations and sources varied with time and were similar to almost all clam farms. The highest PAH concentration was in the winter and the abundance of PAHs with 4 to 6 aromatic rings in these samples indicates that they were most likely associated with domestic heating, urban run-off or river discharges which are usually greatest in periods of abundant rainfall like the one registered at the time of this study (http://snirh.inag.pt; Launhardt & Thoma, 2000; McCready *et al.*, 2000; Marttinen *et al.*, 2003; Mitra & Bianchi, 2003; Prevedouros *et al.*, 2003; Zou *et al.*, 2003). PAH

concentrations decreased gradually from winter to summer and August presented the lowest PAH concentrations from a mixed petrogenic and pyrogenic source, namely oil, soot, creosote and tar (Figure 2.12). The oil signature is usually related to oil spills. Although no severe oil spills have occurred in this area, at least eleven accidental or deliberate hydrocarbon releases were reported in the Ria Formosa and/or South Coast of Portugal in this period (CCDR, 2005). Insidious pollution is also a known PAHs source to the marine environment (Vieites *et al.*, 2004). The South Portuguese coast is located close to the main routes of large oil tankers going in and out of the Mediterranean Sea, which could also contribute to the appearance of this type of signature in the Ria Formosa. The other signatures are generally related to boats (Burns *et al.*, 1997; Page *et al.*, 1999). Boats are considered an important source of pollutants, including PAHs, to the marine environment since most boats and ships (including other watercrafts as Jet Skis) emit part of their fuel/oil mixture and exhaust gases into the water (Kado *et al.*, 2000; Kelly *et al.*, 2005). Although, it is impossible to determine the exact number of boats inside the Ria Formosa, there is a large increase (around 10x) in boat traffic in the summer due to tourism and fishing activities. In this way, it could be roughly estimated that 2000 boats (the capacity of Olhão harbour) with an average 75 horse-power engine (55.2 kW) operating at 60% throttle will emit ≈100 g PAHs h^{-1} into the environment. However, without the knowledge of each boat specific characteristics, speed and amount of time spent in the lagoon it is very difficult to quantify the exact amount of PAHs released by boat traffic into this aquatic environment.

PAH concentrations measured in the whole soft tissues in the clam *R. decussatus* were generally low (Figure 3.2; Table A4), when compared with those found in other edible clams (*Tapes philippinarum* and *Venus gallina*) from different European locations (Binelli & Provini, 2003) or in *R. decussatus* from the Galicia Coast (Porte *et al.*, 2000). However, in the summer tPAH concentrations, in some areas, were higher than in clams affected by the Aegean Sea oil spill (Porte *et al.*, 2000).

Similarly to the sediments, spatial and seasonal variation of both tPAH and individual PAH concentrations in clam tissues revealed that seasonal factors prevail over spatial ones (Figures 3.6 and 3.7). PAH concentrations in clams were, opposite to the sediments, higher in the summer and in the winter (August and January). These changes were not related to the variation in the lipid content of clam tissues. In fact, the maximum lipid percentage in the clam tissues occurred in May probably related with the reproduction cycle when PAH concentrations were low (Figure 3.4). Seasonal variations in PAH concentrations were probably related to environmental PAH load which in the summer is likely to be forest fires, human activities and boat traffic (Figure 3.8). In fact, forest fires are common in the south of Portugal in the summer and the population around the Ria Formosa dramatically increases in the summer months due to tourism (Instituto da Água, 1994; Mudge & Bebianno, 1997) which might be responsible for the raise in PAH emissions. In the winter, oil contamination is the most probable PAHs source. PAH levels in clam tissues indicated that there is a change in source in the lagoon throughout the year ranging from a petrogenic to a pyrolytic source and a mixture of both.

Individual PAH distribution patterns in clam tissues may be influenced by accumulation pathways. The main accumulation pathway of organotin compounds in clams is the water (Coelho *et al.*, 2002a; 2002b) and PAHs solubility, as hydrophobic contaminants, is inversely related to K_{ow}. Thus, the more soluble PAHs are generally more bioavailable to the clams than the less soluble ones. However, clams are suspension feeding bivalves and a significant part of the less soluble PAHs is generally associated to particles, especially of organic origin as food particles which might be an important accumulation pathway for the high molecular weight and less soluble PAHs (Kirso *et al.*, 2001). Similarly, the more soluble PAHs are more easily eliminated by bivalve molluscs than the high molecular weight PAHs because the first may be eliminated through passive elimination and the others need to be metabolised in order to be eliminated from the cells (Neff *et al.*, 1976).

Possible implications to human health due to the consumption of these bivalves were also assessed. The large majority of clams were safe for human consumption (Figure 3.9). Some samples, however, presented PAH levels exceeding those considered safe for human consumption and corresponded to samples characterized by high predominance of the 5+6 aromatic rings PAHs, which are the most carcinogenic (Gilroy, 2000). Thus, because these are considered priority substances by the Portuguese legislation and by the Water Framework Directive, further monitoring of PAH concentrations in shellfish from the Ria Formosa should be implemented.

The clam PAH concentrations were related to the sediment PAHs content with the exception of the higher PAH accumulation in clam tissues in August (Figure 3.10), suggesting that, in this period, the sediments were not the main source of PAHs to the clams, reinforcing the importance of the water and/or food as the most predominant pathway. Biota Sediment Accumulation Factors (BSAF; Table A5) were much higher than in the literature (Baumard *et al.*, 1999a; Nasci *et al.*, 1999) confirming that sediments were less representative of the environmental PAHs load than the clams. This could be the result of the amount of surface sediment sampled (top 5 cm), which may have prevented the identification of sudden changes in PAHs concentrations. It may also be the result of the disturbance to which these sediments are subject to as a consequence of the activities related to the clam harvesting.

Having studied the PAH concentrations and distribution patterns in the clam whole soft tissues, in Chapter 4 the variation of the antioxidant enzymes activity and lipid peroxidation products (LPO) in the clam digestive gland was analysed trying to establish the effect of PAH concentrations in this clam tissue.

Like for the PAHs in clam tissues, the seasonal variation of antioxidant enzymes and LPO also prevailed over the spatial variability but was enzyme dependent. SOD in the mitochondrial fraction (Mit SOD) is a Mn-SOD having the specific role of dismutating the

superoxide anion generated in the respiratory chain (Matés, 2000). The most abundant form of SOD, however, is the Cu/Zn-SOD (70% of the total SOD) which is located in the cells cytosol (Cyt SOD) (Livingstone *et al.*, 1992; Orbea *et al.*, 2000; Geret *et al.*, 2003; Geret & Bebianno, 2004). Mit SOD activity was highest in the summer and lowest in the winter and spring while, on the contrary, Cyt SOD was more elevated in autumn and presented lower activities in the summer. However, these enzyme activities (Mit and Cyt SOD) were not affected by PAHs accumulated in the clam tissues (Figure 4.6; Table A9) suggesting that these two enzymes are responding differently to the environmental conditions to which clams are exposed to (Figure 4.1). Although a direct relationship exists between SOD activity and PAHs in other bivalve molluscs (*Mytilus* sp.) as a response to increased PAH concentrations (Solé *et al.*, 1994; Solé *et al.*, 1995), decreases or no variation of SOD activity was also reported for *M. galloprovincialis* exposed to PAHs or to other contaminants as PCBs, DDT or lindane (Videla *et al.*, 1990; Livingstone *et al.*, 1995; Orbea *et al.*, 2002). The lack of SOD induction indicates the efficiency in oxyradical scavenging by non-enzymatic dietary antioxidants, as vitamins E and C, glutathione, carotenoids or flavonoids, which constitute also important antioxidant defences (Halliwell, 1996; Lesser, 2006).

CAT activity showed both seasonal and spatial variation (highest in spring and lower in summer; Figure 4.2) which could be linked to an increase of oxyradicals production associated with higher metabolic rates during this season (spring) as a preparation for reproduction (Orbea *et al.*, 1999; Viarengo *et al.*, 1991). In fact, the seasonal variation of CAT activity was followed by similar responses in LPO, pointing to an increase in oxidative stress. Contrary to SOD, CAT was negatively related to PAH concentrations (Figure 4.6; Table A9), however, this could be related to the rate of CAT turnover which can be reduced under stressful conditions such as the high water temperatures usually measured in the Ria Formosa during summer (Chícharo & Chícharo, 2001a; Lesser, 2006).

Total glutathione peroxidase (T GPx) and Selenium dependent glutathione peroxidase (Se GPx) activities (Figures 4.3 and 4.4) presented a general decreasing trend with increasing PAH concentrations (Figure 4.6; Table A9) which indicate a precarious state of the clams associated with PAHs toxicity (Cossu *et al.*, 2000).

Similarly to CAT and T and Se GPx, LPO in the clam digestive gland (Figure 4.5) was inversely related to the PAH concentrations in the clam tissues, indicating that the increases in PAH concentrations in the clam tissues were not leading to membrane oxidative damage in *R. decussatus* digestive gland.

The seasonal variation in these oxidative stress related markers, may also be related to temperature (Regoli, 1998; Fisher *et al.*, 1999; Vidal *et al.*, 2002a), oxygen concentration (Speit *et al.*, 2002), exposure to UV radiation (Vega & Pizarro, 2000; Estevez *et al.*, 2001), parasitism (Dautremepuits *et al.*, 2003) or the reproductive status of the animal (Cancio *et al.*, 1999; Sheehan & Power, 1999). Thus, the seasonal variation in the antioxidant enzymes activity and LPO are probably the result of a combined effect of different contaminants and environmental conditions in the Ria Formosa through out the year.

Therefore, it was not possible to establish a direct cause-effect relationship between PAHs in the whole soft tissues and antioxidant enzymes activity or lipid peroxidation products in clams digestive gland. Although it has been established that PAHs are redox cycling compounds, which may act as pro-oxidants, on a global scale several studies have described variations in antioxidant enzymes activity and LPO in bivalve molluscs induced by other environmental contaminants such as metals (Geret *et al.*, 2002a; 2002b; Geret & Bebianno, 2004; Bebianno *et al.*, 2004) or PCBs (Krishnakumar *et al.*, 1997; Orbea *et al.*, 2002; Rodríguez-Ariza *et al.*, 2003).Thus, the effect of PAHs may be masked by the presence of these compounds in the Ria Formosa (Castro & Vale, 1995; Ferreira & Vale, 1995, 1998; Bebianno & Machado, 1997; Bebianno & Serafim, 2003).

Since the relationship between PAHs and antioxidant enzymes was not clear in the seasonal and spatial study (Chapter 4), an active biomonitoring experiment was planned (Chapter 5) in which PAH concentrations and antioxidant enzymes, LPO and two enzymes from phase I and II of organic xenobiotics metabolism (BPH and GST) were studied. This experiment was performed in winter-spring in order to clarify the mechanism of PAHs accumulation, their metabolisation and their effects in antioxidant enzymes and LPO in *R. decussatus* and proceeded in two phases: i) clams were transplanted between two sites (sites 5 and 7) with significantly different PAH concentrations in sediment and clams during the seasonal and spatial study between January and May (Chapters 2 and 3) and ii) were afterwards backtransplanted to the original site.

During the transplant experiment PAHs were significantly accumulated by clams at both sites, on the first day of the experiment (Figure 5.1) although the accumulation was higher at site 7. The individual PAHs distribution in clam tissues was site dependent indicating different PAHs sources on both sites (Figures 5.2 and 5.3). Acenaphthene and benzo[*a*]anthracene were the most accumulated PAHs at both sites reflecting clams preferential uptake of contaminants through the water-phase (Sericano *et al.*, 1996; Neff, 2002). Afterwards, PAHs elimination occurred continuously in transplanted clams but not at site 5. In clams at this site, PAH concentrations fluctuated and by the end of the experiment PAH levels were higher than at the transplant site reflecting different PAH sources to both locations.

Similarly, despite the differences in PAH levels (Figure 5.1), most of the enzymes were site dependent probably because PAH concentrations were not enough to induce distinct levels of biomarker responses. The activities of GST, SOD and CAT were significantly higher in the gills confirming previous data (Geret *et al.*, 2003) representing the first line of defence against exposure or damage to the more soluble PAHs (Ac was the most accumulated PAH) (Cossu *et al.*, 2000; Cheung *et al.*, 2001). On the contrary, BPH activity was only detected in the digestive

gland and LPO was also significantly higher in this tissue (Figure 5.8) indicating that this organ is probably the main responsible for PAHs metabolism.

Biomarker responses were tissue dependent. In the gills, Cyt SOD, T and Se GPx and GST were directly related to Ac and BaA (Figure 5.11) indicating induction of oxidative stress by these PAHs. In this case, GST appears to act as a peroxidase since the activity BPH was not detected in the gills (Varanasi *et al.*, 1989; Sheehan *et al.*, 2001). Similar induction of T GPx and GST in relation to PAHs exposure was already reported in the gills of *U. tumidus* transplanted between different contaminated sites in Hong Kong (Cheung *et al.*, 2001). In the digestive gland, Mit SOD, BPH and GST were also positively related with Ac and BaA (Figure 5.11). This indicates that PAHs metabolism (Ac and BaA in particular) is an active process in this tissue since BPH and GST are involved in phases I and II of organic xenobiotics metabolism, respectively (Peters *et al.*, 1999; Hoarau *et al.*, 2001). Mit SOD induction indicates that PAHs metabolism is also leading to the production of oxyradicals (superoxide anion in particular) probably through the redox cycling of BPH activity products (Fabiani *et al.*, 1999). The production of oxyradicals, associated with a decrease in most antioxidant enzyme activities after day 7 lead to oxidative damage translated by an increase in lipid peroxidation products in the digestive gland which was highest at day 14 (Figure 5.8).

The other antioxidant enzyme activities although changing during the transplant experiment were apparently not related to the PAHs accumulated in clam tissues.

During the backtransplant experiment, the variation of PAH concentrations was smaller than during the transplant experiment (Figure 5.4). However, despite the fact that both clam groups were now exposed to the same conditions, clams backtransplanted from site 7 to site 5 accumulated PAHs differently than those remaining at site 5 and were not able to acclimate to the new environmental conditions during the period of this study. Similarly, biomarkers were also less variable than in the transplant experiment but were also tissue dependent (Figures 5.9

and 5.10). The most relevant feature was the opposite trend of the biomarker variations with PAHs between tissues (Figure 5.13) pointing once more to a different function of these organs. In the gills, most markers (especially Cyt SOD and T GPx) were negatively related to the PAHs (especially, A, P, Fluo and Ch) similarly to what occurred in the mussel *U. tumidus* transplanted to a PAHs and metals contaminated site (Cossu *et al.*, 1997). In the digestive gland, BPH, GST and CAT were the most relevant biomarkers presenting higher explained variances (Figure 5.14) confirming the role ofBPH and GST in PAHs (Ac and BaA in particular) metabolism.

In conclusion:

i) Seasonal variations of PAH concentrations and sources in both sediments and clam whole soft tissues in the Ria Formosa prevail over spatial variation;

ii) PAH concentrations in the sediments were generally lower than the ecotoxicological assessment criteria defined for surface sediments by the OSPAR Commission (OSPAR Commission, 2000);

iii) PAHs in clams were low, though in the summer high PAH concentrations exist in some places which raises concern relatively to the human consumption of this shellfish indicating the need for further monitoring of PAHs levels in the Ria Formosa clams;

iv) Several PAH sources were identified which changed with season: domestic heating, urban run-off and river discharges associated with periods of intense rainfall in the winter (more important in the sediments) and boat traffic, human activities and forest fires in the summer (common to both sediments and clams);

v) Though a good correlation between sediment and clam PAHs exist, in the summer (August) PAH concentrations were much higher in clams than in sediments indicating the importance of water and/or food accumulation pathways instead of sediments;

vi) Antioxidant enzyme activities in the digestive gland changed seasonally rather than spatially but these changes were not always related to the seasonal variation in clam PAHs content;

vii) Mit and Cyt SOD were not related to PAHs;

viii) CAT was strongly affected by seasonal factors like the reproductive cycle and water temperature;

ix) T and Se GPx decreased due to PAHs induced toxicity;

x) LPO was also negatively related with the PAHs indicating the absence of oxidative damage in the clam digestive gland induced by PAHs;

xi) In the transplant experiment between two clam farms, significant PAHs accumulation was observed in the clam whole soft tissues followed by the induction of BPH and GST in the digestive gland emphasizing PAHs metabolisation by *R. decussatus*;

xii) PAHs exposure and metabolisation induced oxidative stress in both clam tissues translated by increases of Cyt SOD and T GPx in the gills and Mit SOD and CAT in the digestive gland, and in LPO products although more important in the digestive gland.

It is generally established that a useful biomarker should be inducible by environmentally realistic concentrations of pollutants; produce clear dose responses to those pollutant concentrations; and present little seasonal variations. Most of the antioxidant enzymes studied in the digestive gland of *R. decussatus* from the Ria Formosa fail to accomplish some of these conditions. Namely: i) Mit and Cyt SOD were not related with PAH concentrations (Chapter 4) and were not always induced in the transplant experiment (Chapter 5); ii) CAT changed seasonally probably due to the clam reproductive cycle (Chapter 4) and failed to respond to PAHs accumulation in the transplant study (Chapter 5); and iii) T and Se GPx though negatively related to PAHs (Chapter 4) were not always related to PAHs in the transplant study. Thus,

antioxidant enzymes are not considered effective biomarkers of PAHs exposure in *R. decussatus*. BPH, GST and LPO in the digestive gland, however, were induced following PAHs exposure but their seasonal variation in *R. decussatus* is not known and should be studied before they can be employed in environmental monitoring studies.

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ANNEXE

Table A1 – Water temperature (ºC).

	August I	October	November	December	January	March	May	June	July	August II
Site 1	22.3	19.9	17.7	18.4	15.2	16.0	18.3	19.1	24.2	23.1
Site 2	23.5	18.2	17.9	18.1	15.2	n.a.	17.8	n.a.	n.a.	24.3
Site 3	n.a.	18.7	18.2	17.7	15.2	n.a.	18.6	n.a.	n.a.	24.8
Site 4	19.9	18.1	17.0	18.5	15.7	16.7	21.8	20.4	19.3	27.8
Site 5	20.9	18.4	16.5	19.3	16.0	n.a.	18.3	n.a.	n.a.	26.5
Site 6	22.7	19.1	16.3	20.0	15.0	15.9	20.6	21.6	26.5	n.a.
Site 7	20.8	18.8	20.4	18.4	15.3	15.6	18.7	19.0	24.7	25.3
Site 8	23.1	20.9	n.a.	18.5	15.2	n.a.	22.4	n.a.	n.a.	28.1

n.a. – data not available.

Table A2 – Total and individual Polycyclic Aromatic Hydrocarbons (PAHs) concentrations (ng g^{-1} d.w.) in the sediments from different sites in the Ria Formosa lagoon.

Site / PAHs	1	2	3	4	5	6	7	8
January								
N	0.53 ± 0.16	0.40 ± 0.03	0.47 ± 0.18	n.d.	0.32 ± 0.06	n.d.	0.02 ± 0.00	n.d.
Ace	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ac	5.51 ± 0.51	2.68 ± 0.78	2.81 ± 1.07	1.65 ± 0.39	4.39 ± 1.35	13.3 ± 0.5	11.5 ± 0.7	6.01 ± 0.74
F	1.64 ± 0.20	0.52 ± 0.06	0.66 ± 0.17	0.85 ± 0.06	0.54 ± 0.03	1.01 ± 0.16	1.01 ± 0.23	0.48 ± 0.06
P	3.17 ± 0.45	0.70 ± 0.06	0.98 ± 0.13	0.43 ± 0.06	1.61 ± 0.33	1.35 ± 0.10	1.92 ± 0.31	0.35 ± 0.03
A	0.83 ± 0.09	0.09 ± 0.02	0.24 ± 0.09	0.16 ± 0.03	0.64 ± 0.09	0.11 ± 0.00	0.58 ± 0.16	0.09 ± 0.02
Fluor	9.90 ± 1.22	1.68 ± 0.06	1.68 ± 0.53	0.24 ± 0.07	5.06 ± 1.65	0.79 ± 0.10	6.51 ± 0.56	2.97 ± 0.65
Py	5.87 ± 0.08	3.30 ± 0.77	1.24 ± 0.22	2.18 ± 0.11	4.70 ± 0.97	1.97 ± 0.48	4.20 ± 1.32	2.19 ± 0.18
BaA	4.47 ± 0.35	2.04 ± 0.62	1.45 ± 0.23	0.49 ± 0.10	2.48 ± 0.10	4.15 ± 0.27	4.79 ± 0.36	1.87 ± 0.65
Chr	1.98 ± 0.45	0.47 ± 0.03	0.53 ± 0.08	n.d.	0.45 ± 0.00	2.54 ± 0.14	4.19 ± 0.05	1.73 ± 0.58
BbF	1.71 ± 0.14	n.d.	0.95 ± 0.31	0.17 ± 0.04	1.28 ± 0.52	5.41 ± 0.46	n.d.	1.82 ± 0.68
BkF	1.47 ± 0.36	n.d.	0.26 ± 0.01	0.15 ± 0.01	n.d.	3.20 ± 0.09	n.d.	0.30 ± 0.06
BaP	6.22 ± 1.12	n.d.	0.27 ± 0.02	0.02 ± 0.00	n.d.	0.92 ± 0.05	2.50 ± 0.09	n.d.
DBahA	n.d.	0.08 ± 0.01	0.01 ± 0.00	n.d.	0.47 ± 0.19	26.5 ± 6.3	5.11 ± 0.25	0.46 ± 0.09
BghiP	n.d.	n.d.	n.d.	0.01 ± 0.00	n.d.	4.14 ± 0.52	n.d.	n.d.
IP	n.d.	n.d.	n.d.	0.01 ± 0.00	n.d.	0.99 ± 0.06	n.d.	0.28 ± 0.06
tPAH	**43.3 ± 1.9**	**12.0 ± 1.3**	**11.6 ± 1.3**	**6.35 ± 0.43**	**21.9 ± 2.4**	**66.3 ± 6.4**	**42.3 ± 1.7**	**18.5 ± 1.5**
March								
N	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
Ace	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
Ac	8.27 ± 1.82	n.a.	n.a.	n.d.	n.a.	3.49 ± 0.95	n.d.	n.a.
F	2.44 ± 0.25	n.a.	n.a.	n.d.	n.a.	0.63 ± 0.07	n.d.	n.a.
P	1.74 ± 0.02	n.a.	n.a.	0.26 ± 0.05	n.a.	0.52 ± 0.07	0.21 ± 0.01	n.a.
A	0.22 ± 0.02	n.a.	n.a.	0.05 ± 0.00	n.a.	0.10 ± 0.03	0.05 ± 0.01	n.a.
Fluor	1.10 ± 0.05	n.a.	n.a.	0.29 ± 0.02	n.a.	0.33 ± 0.08	0.20 ± 0.03	n.a.
Py	0.56 ± 0.02	n.a.	n.a.	1.69 ± 0.30	n.a.	0.51 ± 0.11	0.38 ± 0.10	n.a.
BaA	1.15 ± 0.13	n.a.	n.a.	0.35 ± 0.03	n.a.	0.67 ± 0.08	0.35 ± 0.02	n.a.
Chr	0.84 ± 0.04	n.a.	n.a.	0.32 ± 0.02	n.a.	0.27 ± 0.05	0.10 ± 0.03	n.a.
BbF	0.35 ± 0.01	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
BkF	0.24 ± 0.07	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
BaP	0.05 ± 0.01	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
DBahA	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
BghiP	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
IP	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
tPAH	**17.0 ± 1.8**			**2.96 ± 0.30**		**6.51 ± 0.97**	**1.29 ± 0.11**	
May								
N	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ace	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ac	9.47 ± 2.33	2.02 ± 0.43	1.13 ± 0.02	1.87 ± 0.13	2.46 ± 0.62	3.27 ± 0.62	1.64 ± 0.22	2.77 ± 0.71
F	1.76 ± 0.11	0.39 ± 0.08	0.30 ± 0.08	0.32 ± 0.08	0.41 ± 0.05	0.49 ± 0.05	0.22 ± 0.10	0.33 ± 0.08
P	0.90 ± 0.00	0.24 ± 0.03	0.45 ± 0.03	0.23 ± 0.03	0.23 ± 0.02	0.61 ± 0.14	0.44 ± 0.08	0.24 ± 0.03
A	0.09 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	0.07 ± 0.02	0.06 ± 0.01	0.05 ± 0.01
Fluor	0.98 ± 0.25	0.49 ± 0.05	0.42 ± 0.02	0.21 ± 0.03	0.18 ± 0.00	0.86 ± 0.11	0.25 ± 0.07	0.19 ± 0.02
Py	0.75 ± 0.15	0.37 ± 0.06	0.41 ± 0.05	0.09 ± 0.01	0.10 ± 0.00	0.65 ± 0.15	0.25 ± 0.15	0.16 ± 0.03
BaA	0.54 ± 0.02	0.36 ± 0.01	0.41 ± 0.02	0.23 ± 0.02	0.08 ± 0.00	0.74 ± 0.06	0.33 ± 0.22	0.25 ± 0.03
Chr	0.25 ± 0.04	0.19 ± 0.01	0.22 ± 0.03	0.13 ± 0.02	0.04 ± 0.01	0.30 ± 0.04	0.24 ± 0.20	0.24 ± 0.01
BbF	0.15 ± 0.01	0.12 ± 0.00	0.20 ± 0.05	n.d.	n.d.	n.d.	0.11 ± 0.03	n.d.
BkF	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.02 ± 0.00	n.d.
BaP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.01 ± 0.01	n.d.
DBahA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BghiP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
IP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
tPAH	**14.9 ± 2.3**	**4.25 ± 0.45**	**3.60 ± 0.12**	**3.12 ± 0.16**	**3.58 ± 0.62**	**6.98 ± 0.67**	**3.57 ± 0.42**	**4.24 ± 0.72**
June								
N	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
Ace	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
Ac	n.d.	n.a.	n.a.	5.16 ± 0.00	n.a.	4.55 ± 0.54	n.d.	n.a.
F	n.d.	n.a.	n.a.	0.70 ± 0.00	n.a.	0.29 ± 0.09	0.18 ± 0.05	n.a.
P	0.32 ± 0.11	n.a.	n.a.	1.04 ± 0.00	n.a.	1.25 ± 0.31	0.82 ± 0.19	n.a.
A	0.05 ± 0.01	n.a.	n.a.	0.10 ± 0.00	n.a.	0.13 ± 0.04	0.08 ± 0.01	n.a.
Fluor	0.33 ± 0.02	n.a.	n.a.	0.56 ± 0.00	n.a.	0.40 ± 0.06	0.46 ± 0.04	n.a.
Py	0.35 ± 0.02	n.a.	n.a.	0.66 ± 0.00	n.a.	0.45 ± 0.09	0.48 ± 0.12	n.a.
BaA	0.24 ± 0.13	n.a.	n.a.	0.62 ± 0.00	n.a.	0.73 ± 0.06	0.46 ± 0.09	n.a.
Chr	0.17 ± 0.00	n.a.	n.a.	0.37 ± 0.00	n.a.	0.14 ± 0.03	0.17 ± 0.03	n.a.
BbF	n.d.	n.a.	n.a.	n.d.	n.a.	0.08 ± 0.01	0.09 ± 0.02	n.a.
BkF	0.06 ± 0.02	n.a.	n.a.	n.d.	n.a.	0.01 ± 0.00	0.24 ± 0.05	n.a.
BaP	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
DBahA	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
BghiP	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
IP	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
tPAH	**1.52 ± 0.18**			**9.21 ± 0.00**		**8.01 ± 0.64**	**2.99 ± 0.25**	

Table A2 (cont.)

Site / PAHs	1	2	3	4	5	6	7	8
July								
N	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
Ace	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
Ac	n.d.	n.a.	n.a.	0.99 ± 0.23	n.a.	n.d.	n.d.	n.a.
F	0.32 ± 0.08	n.a.	n.a.	0.18 ± 0.01	n.a.	n.d.	0.85 ± 0.13	n.a.
P	0.14 ± 0.04	n.a.	n.a.	0.27 ± 0.04	n.a.	0.34 ± 0.05	0.82 ± 0.07	n.a.
A	0.04 ± 0.01	n.a.	n.a.	0.05 ± 0.01	n.a.	0.05 ± 0.01	0.13 ± 0.01	n.a.
Fluor	0.14 ± 0.02	n.a.	n.a.	0.18 ± 0.04	n.a.	0.23 ± 0.07	0.26 ± 0.05	n.a.
Py	0.10 ± 0.01	n.a.	n.a.	0.18 ± 0.04	n.a.	0.13 ± 0.01	0.14 ± 0.03	n.a.
BaA	0.15 ± 0.04	n.a.	n.a.	0.19 ± 0.04	n.a.	0.13 ± 0.01	0.35 ± 0.02	n.a.
Chr	0.10 ± 0.01	n.a.	n.a.	0.10 ± 0.00	n.a.	0.12 ± 0.03	0.16 ± 0.01	n.a.
BbF	0.09 ± 0.01	n.a.	n.a.	n.d.	n.a.	n.d.	0.06 ± 0.01	n.a.
BkF	n.d.	n.a.	n.a.	n.d.	n.a.	0.10 ± 0.02	0.11 ± 0.01	n.a.
BaP	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	0.01 ± 0.00	n.a.
DBahA	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
BghiP	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
IP	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
tPAH	**1.08 ± 0.10**			**2.14 ± 0.24**		**1.10 ± 0.10**	**2.87 ± 0.16**	
August								
N	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.
Ace	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.
Ac	n.d.	0.44 ± 0.05	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.
F	0.36 ± 0.02	0.31 ± 0.08	0.37 ± 0.08	n.d.	0.18 ± 0.03	n.a.	n.d.	0.51 ± 0.01
P	0.27 ± 0.03	0.40 ± 0.10	0.39 ± 0.16	0.58 ± 0.03	0.26 ± 0.02	n.a.	0.42 ± 0.03	0.50 ± 0.06
A	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.13 ± 0.02	0.06 ± 0.01	n.a.	0.06 ± 0.01	0.07 ± 0.01
Fluor	0.11 ± 0.01	0.07 ± 0.00	0.16 ± 0.04	0.62 ± 0.16	0.20 ± 0.02	n.a.	0.20 ± 0.06	0.25 ± 0.01
Py	0.09 ± 0.00	0.13 ± 0.02	0.08 ± 0.01	0.22 ± 0.02	0.16 ± 0.01	n.a.	0.11 ± 0.02	0.17 ± 0.02
BaA	0.17 ± 0.02	0.22 ± 0.06	0.05 ± 0.01	0.16 ± 0.04	0.08 ± 0.02	n.a.	0.15 ± 0.03	0.25 ± 0.03
Chr	0.11 ± 0.02	0.15 ± 0.04	0.05 ± 0.01	0.09 ± 0.02	0.07 ± 0.01	n.a.	0.07 ± 0.00	0.09 ± 0.02
BbF	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.
BkF	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.
BaP	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.
DBahA	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.
BghiP	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.
IP	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.
tPAH	**1.18 ± 0.05**	**1.78 ± 0.15**	**1.14 ± 0.18**	**1.78 ± 0.17**	**1.01 ± 0.06**		**1.01 ± 0.07**	**1.84 ± 0.07**

n.d. – not detected; n.a. – data not available.

Table A3 – Ecotoxicological Assessment Criteria for PAHs in superficial sediments.

Compound	Sediment ($\mu g\ g^{-1}$ d.w.)
Naphthalene	0.05-0.5
Phenanthrene	0.1-1
Anthracene	0.05-0.5
Fluoranthene	0.5-5
Pyrene	0.05-0.5
Benzo[*a*]anthracene	0.1-1
Chrysene	0.1-1
Benzo[*k*]fluoranthene	nd
Benzo[*a*]pyrene	0.1-1
Benzo[*ghi*]perylene	nd
Indene[123-*cd*]pyrene	nd

Adapted from OSPAR, 2000.

Table A4 – PAHs concentrations (ng g^{-1} w.w.) in the clam whole soft tissues.

Site / PAHs	1	2	3	4	5	6	7	8
August I								
N	4.53 ± 0.80	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.
Ace	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.
Ac	33.2 ± 10.0	30.6 ± 6.0	n.a.	83.4 ± 17.2	n.d.	2.37 ± 0.22	74.1 ± 8.5	n.d.
F	8.11 ± 0.98	15.7 ± 3.3	n.a.	5.92 ± 1.51	5.97 ± 1.64	3.23 ± 0.43	7.34 ± 1.79	2.33 ± 0.00
P	19.0 ± 2.4	40.7 ± 14.4	n.a.	18.8 ± 4.3	11.5 ± 0.8	12.4 ± 1.1	44.4 ± 0.0	15.0 ± 3.7
A	3.51 ± 0.54	8.58 ± 2.95	n.a.	2.96 ± 0.68	1.97 ± 0.11	1.69 ± 0.55	6.58 ± 0.29	2.14 ± 0.64
Fluor	51.7 ± 7.1	55.6 ± 13.8	n.a.	38.9 ± 10.9	25.2 ± 3.7	31.7 ± 2.4	110 ± 19	33.5 ± 7.7
Py	28.6 ± 4.6	114 ± 23	n.a.	21.6 ± 5.0	12.5 ± 1.4	25.0 ± 5.1	138 ± 28	23.9 ± 4.8
BaA	83.0 ± 11.8	275 ± 71	n.a.	28.9 ± 3.4	23.8 ± 4.0	41.5 ± 5.3	241 ± 22	24.9 ± 3.8
Chr	50.7 ± 6.1	121 ± 38	n.a.	27.2 ± 2.9	13.5 ± 2.8	29.5 ± 8.8	113 ± 7	18.2 ± 2.6
BbF	11.5 ± 1.4	104 ± 33	n.a.	n.d.	n.d.	12.7 ± 3.2	n.d.	n.d.
BkF	6.85 ± 2.24	n.d.	n.a.	3.62 ± 0.87	1.73 ± 0.31	7.51 ± 5.51	n.d.	6.47 ± 0.55
BaP	n.d.	135 ± 25	n.a.	n.d.	n.d.	n.d.	122 ± 30	n.d.
DBahA	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.
BghiP	n.d.	n.d.	n.a.	1.11 ± 0.21	n.d.	n.d.	n.d.	n.d.
IP	n.d.	13.6 ± 2.4	n.a.	0.46 ± 0.13	n.d.	n.d.	49.0 ± 2.0	n.d.
tPAH	**301 ± 40**	**914 ± 170**		**233 ± 11**	**96.2 ± 9.1**	**168 ± 13**	**906 ± 9**	**126 ± 23**
October								
N	n.d.	n.d.	n.d.	6.97 ± 0.82	n.d.	n.d.	n.d.	3.39 ± 0.40
Ace	n.d.	58.5 ± 17.0	58.3 ± 0.2	33.3 ± 2.4	n.d.	n.d.	3.03 ± 0.57	7.46 ± 2.27
Ac	24.4 ± 0.0	120 ± 16	50.1 ± 9.9	24.0 ± 1.2	18.0 ± 2.5	19.2 ± 0.7	14.0 ± 3.1	11.3 ± 3.1
F	9.96 ± 0.00	n.d.	5.34 ± 0.27	0.33 ± 0.06	1.84 ± 0.30	2.11 ± 0.10	1.73 ± 0.31	2.64 ± 0.39
P	3.39 ± 0.00	4.73 ± 0.80	3.74 ± 0.34	1.61 ± 0.16	9.31 ± 2.73	64.6 ± 7.3	28.6 ± 3.6	5.22 ± 0.55
A	0.44 ± 0.00	1.46 ± 0.41	1.38 ± 0.15	0.97 ± 0.13	0.61 ± 0.03	0.84 ± 0.15	0.70 ± 0.17	2.77 ± 0.89
Fluor	3.42 ± 0.00	14.1 ± 3.5	13.1 ± 0.5	12.2 ± 0.8	13.7 ± 1.3	15.9 ± 2.7	6.12 ± 0.82	12.7 ± 2.9
Py	n.d.	8.10 ± 1.03	5.80 ± 1.03	3.07 ± 0.04	13.8 ± 4.1	15.6 ± 0.1	8.66 ± 2.29	10.1 ± 3.0
BaA	8.50 ± 0.00	9.63 ± 0.26	4.77 ± 0.47	4.21 ± 1.52	47.6 ± 6.9	58.7 ± 11.5	15.8 ± 0.4	35.3 ± 8.2
Chr	11.6 ± 0.0	7.74 ± 1.22	2.99 ± 0.98	2.01 ± 0.39	27.4 ± 3.4	32.0 ± 3.3	9.36 ± 1.69	24.4 ± 5.9
BbF	8.17 ± 0.00	8.64 ± 0.95	5.09 ± 1.37	1.85 ± 0.39	9.97 ± 1.55	17.2 ± 4.3	7.85 ± 2.13	14.9 ± 2.4
BkF	3.86 ± 0.00	5.24 ± 1.08	3.36 ± 0.58	0.93 ± 0.16	8.54 ± 1.63	8.10 ± 0.91	4.74 ± 1.24	6.80 ± 0.60
BaP	n.d.	1.75 ± 0.17	1.02 ± 0.27	0.40 ± 0.06	n.d.	n.d.	3.18 ± 0.13	5.58 ± 0.37
DBahA	n.d.	14.6 ± 1.3	8.74 ± 1.06	0.28 ± 0.07	n.d.	n.d.	0.77 ± 0.03	11.0 ± 0.8
BghiP	n.d.	6.92 ± 1.12	n.d.	n.d.	n.d.	n.d.	0.50 ± 0.09	2.18 ± 0.21
IP	n.d.	1.82 ± 0.22	n.d.	n.d.	n.d.	n.d.	0.37 ± 0.11	0.84 ± 0.04
tPAH	**73.7 ± 0.0**	**263 ± 28**	**164 ± 10**	**92.1 ± 2.8**	**151 ± 4**	**234 ± 27**	**105 ± 13**	**157 ± 17**
November								
N	4.60 ± 0.28	0.31 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.
Ace	n.d.	n.d.	n.d.	n.d.	n.d.	8.66 ± 2.27	n.d.	n.a.
Ac	50.4 ± 13.0	4.13 ± 0.86	0.59 ± 0.02	n.d.	5.53 ± 0.68	27.5 ± 1.6	2.92 ± 4.50	n.a.
F	4.16 ± 0.46	1.68 ± 0.06	0.46 ± 0.04	3.32 ± 0.20	0.97 ± 0.02	1.52 ± 0.10	0.77 ± 0.23	n.a.
P	5.28 ± 0.28	4.41 ± 0.73	4.36 ± 0.81	7.72 ± 1.86	2.93 ± 0.41	3.54 ± 0.07	2.96 ± 0.15	n.a.
A	1.27 ± 0.04	0.70 ± 0.13	0.60 ± 0.11	0.96 ± 0.26	0.34 ± 0.05	0.45 ± 0.07	0.43 ± 0.00	n.a.
Fluor	10.2 ± 0.8	9.97 ± 1.38	12.5 ± 0.2	16.1 ± 3.4	4.59 ± 0.89	6.10 ± 0.30	6.14 ± 0.33	n.a.
Py	6.96 ± 0.61	7.80 ± 1.40	8.79 ± 0.33	6.57 ± 1.58	3.98 ± 0.15	6.51 ± 0.48	4.39 ± 0.37	n.a.
BaA	18.5 ± 2.0	25.1 ± 5.2	11.6 ± 0.9	20.8 ± 4.8	11.4 ± 2.6	20.5 ± 2.1	10.6 ± 1.1	n.a.
Chr	4.68 ± 0.79	11.2 ± 2.1	9.94 ± 0.61	10.7 ± 1.3	6.62 ± 0.46	12.3 ± 0.4	5.92 ± 0.53	n.a.
BbF	n.d.	7.08 ± 1.39	3.64 ± 1.00	5.94 ± 0.68	6.23 ± 0.74	9.84 ± 2.68	2.53 ± 0.77	n.a.
BkF	5.88 ± 0.22	3.25 ± 0.44	2.80 ± 0.31	2.35 ± 2.56	2.18 ± 0.55	2.81 ± 0.36	0.23 ± 0.04	n.a.
BaP	n.d.	n.d.	n.d.	4.74 ± 0.33	1.43 ± 0.63	n.d.	n.d.	n.a.
DBA	n.d.	n.d.	0.01 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.a.
BGHP	n.d.	n.d.	0.18 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.a.
IP	n.d.	n.d.	n.d.	0.02 ± 0.00	0.07 ± 0.00	n.d.	0.02 ± 0.00	n.a.
tPAH	**112 ± 15**	**75.6 ± 12.1**	**55.4 ± 2.7**	**79.2 ± 5.4**	**46.3 ± 3.3**	**99.6 ± 3.6**	**36.9 ± 5.2**	
December								
N	n.d.	n.d.	0.22 ± 0.07	n.d.	0.12 ± 0.04	0.22 ± 0.06	n.d.	0.25 ± 0.07
Ace	n.d.	n.d.	n.d.	2.47 ± 0.12	n.d.	1.47 ± 0.13	n.d.	3.83 ± 0.87
Ac	1.04 ± 0.20	5.77 ± 0.64	5.57 ± 0.40	14.6 ± 2.2	n.d.	n.d.	0.39 ± 0.11	0.54 ± 0.05
F	0.51 ± 0.08	1.35 ± 0.29	0.67 ± 0.02	2.07 ± 0.23	1.22 ± 0.30	0.90 ± 0.16	0.45 ± 0.14	0.71 ± 0.10
P	1.79 ± 0.51	5.07 ± 0.23	2.24 ± 0.59	5.00 ± 0.43	4.52 ± 0.25	3.65 ± 0.26	2.41 ± 0.09	0.24 ± 0.02
A	0.20 ± 0.04	0.62 ± 0.08	0.19 ± 0.01	0.61 ± 0.07	0.75 ± 0.17	0.43 ± 0.03	0.27 ± 0.04	0.31 ± 0.01
Fluor	4.70 ± 1.48	12.2 ± 2.4	6.67 ± 0.89	12.0 ± 0.6	10.9 ± 3.0	11.4 ± 1.5	6.28 ± 0.85	6.55 ± 1.75
Py	3.31 ± 0.18	11.3 ± 1.6	7.63 ± 0.82	8.24 ± 0.26	14.4 ± 3.2	9.10 ± 0.75	3.96 ± 0.76	3.91 ± 1.17
BaA	8.49 ± 0.43	21.3 ± 0.7	24.4 ± 2.5	23.7 ± 4.0	19.9 ± 1.3	14.9 ± 4.2	12.7 ± 3.2	20.1 ± 0.2
Chr	5.93 ± 1.42	15.0 ± 2.7	14.4 ± 1.6	11.1 ± 0.3	15.6 ± 4.4	14.9 ± 0.2	6.35 ± 1.24	8.09 ± 2.47
BbF	3.20 ± 1.06	7.46 ± 1.41	4.93 ± 0.25	3.40 ± 0.16	6.67 ± 0.73	10.0 ± 0.5	3.23 ± 0.40	3.36 ± 0.86
BkF	1.26 ± 0.21	5.10 ± 1.02	2.39 ± 0.45	1.81 ± 0.35	2.06 ± 0.38	6.20 ± 0.80	1.01 ± 0.18	0.54 ± 0.04
BaP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
DBahA	n.d.	n.d.	n.d.	n.d.	0.02 ± 0.00	n.d.	n.d.	n.d.
BghiP	n.d.	n.d.	n.d.	0.04 ± 0.01	n.d.	n.d.	n.d.	n.d.
IP	n.d.	n.d.	0.10 ± 0.01	n.d.	0.01 ± 0.00	0.02 ± 0.00	n.d.	0.01 ± 0.00
tPAH	**30.4 ± 3.8**	**85.2 ± 9.2**	**69.4 ± 6.2**	**85.1 ± 5.6**	**76.2 ± 6.4**	**73.3 ± 7.6**	**37.1 ± 6.6**	**48.4 ± 5.6**

Table A4 (Cont.)

PAHs \ Site	1	2	3	4	5	6	7	8
January								
N	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ace	7.32 ± 0.93	53.2 ± 1.6	n.d.	n.d.	n.d.	n.d.	6.50 ± 0.62	n.d.
Ac	55.9 ± 4.1	58.3 ± 9.3	60.0 ± 4.6	22.1 ± 5.4	4.16 ± 0.73	8.96 ± 1.15	5.30 ± 1.07	4.35 ± 0.77
F	5.43 ± 0.24	5.29 ± 0.51	30.3 ± 0.3	2.58 ± 0.37	3.45 ± 0.44	4.30 ± 0.79	5.18 ± 1.12	6.18 ± 1.42
P	18.1 ± 0.2	8.93 ± 0.11	29.7 ± 8.0	11.5 ± 0.6	8.51 ± 0.92	14.4 ± 3.6	17.2 ± 2.5	14.8 ± 1.1
A	2.17 ± 0.16	8.10 ± 1.60	3.46 ± 0.72	1.28 ± 0.22	1.29 ± 0.36	1.34 ± 0.06	2.35 ± 0.42	1.58 ± 0.02
Fluor	8.29 ± 2.15	12.9 ± 0.1	20.0 ± 1.0	13.4 ± 0.1	15.8 ± 2.8	41.4 ± 3.5	25.1 ± 1.1	33.5 ± 4.1
Py	9.39 ± 0.84	46.0 ± 3.0	12.9 ± 0.3	7.61 ± 0.53	16.3 ± 1.7	35.4 ± 1.4	22.6 ± 1.6	27.4 ± 2.0
BaA	67.0 ± 8.5	46.2 ± 8.7	20.8 ± 0.2	61.2 ± 1.7	56.2 ± 7.0	80.7 ± 8.7	199 ± 48	212 ± 11
Chr	47.1 ± 6.3	75.2 ± 8.5	34.0 ± 1.2	45.2 ± 4.5	44.6 ± 7.5	59.4 ± 8.8	142 ± 29	159 ± 6
BbF	23.2 ± 4.2	36.9 ± 1.9	11.1 ± 1.7	17.9 ± 2.5	11.9 ± 1.9	64.1 ± 7.1	37.5 ± 6.9	64.1 ± 9.4
BkF	n.d.	25.5 ± 4.2	12.3 ± 1.1	21.1 ± 2.7	n.d.	n.d.	n.d.	31.2 ± 8.1
BaP	9.64 ± 0.56	4.85 ± 0.20	5.44 ± 0.58	n.d.	n.d.	n.d.	n.d.	n.d.
DBahA	3.33 ± 0.59	n.d.	n.d.	n.d.	1.62 ± 0.03	13.1 ± 1.6	n.d.	n.d.
BghiP	1.33 ± 0.22	1.63 ± 0.28	n.d.	n.d.	n.d.	3.71 ± 0.80	13.7 ± 2.9	n.d.
IP	n.d.	1.14 ± 0.09	0.22 ± 0.04	n.d.	0.33 ± 0.06	0.20 ± 0.01	1.95 ± 0.26	2.13 ± 0.09
tPAH	**258 ± 16**	**384 ± 9**	**240 ± 10**	**204 ± 12**	**164 ± 21**	**327 ± 6**	**479 ± 86**	**556 ± 34**
March								
N	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
Ace	n.d.	n.a.	n.a.	7.17 ± 0.72	n.a.	n.d.	n.d.	n.a.
Ac	4.53 ± 0.53	n.a.	n.a.	7.53 ± 1.99	n.a.	1.84 ± 0.23	2.24 ± 0.02	n.a.
F	1.08 ± 0.13	n.a.	n.a.	2.36 ± 0.26	n.a.	0.48 ± 0.07	0.47 ± 0.03	n.a.
P	0.33 ± 0.09	n.a.	n.a.	4.52 ± 0.46	n.a.	4.39 ± 0.70	1.47 ± 0.26	n.a.
A	0.44 ± 0.04	n.a.	n.a.	0.65 ± 0.18	n.a.	0.26 ± 0.08	0.32 ± 0.04	n.a.
Fluor	10.2 ± 2.8	n.a.	n.a.	14.5 ± 3.0	n.a.	15.1 ± 2.1	4.10 ± 0.58	n.a.
Py	4.80 ± 0.50	n.a.	n.a.	5.65 ± 1.40	n.a.	8.89 ± 0.75	3.94 ± 0.17	n.a.
BaA	20.8 ± 5.4	n.a.	n.a.	30.5 ± 5.7	n.a.	42.5 ± 3.0	16.4 ± 0.5	n.a.
Chr	10.8 ± 3.0	n.a.	n.a.	14.2 ± 2.6	n.a.	23.8 ± 0.8	9.91 ± 0.65	n.a.
BbF	3.54 ± 0.64	n.a.	n.a.	5.23 ± 0.71	n.a.	4.99 ± 0.36	2.81 ± 0.41	n.a.
BkF	2.00 ± 0.46	n.a.	n.a.	4.86 ± 0.33	n.a.	9.45 ± 0.16	2.67 ± 0.23	n.a.
BaP	n.d.	n.a.	n.a.	0.89 ± 0.18	n.a.	5.69 ± 0.58	n.d.	n.a.
DBahA	n.d.	n.a.	n.a.	n.d.	n.a.	0.11 ± 0.02	n.d.	n.a.
BghiP	0.04 ± 0.01	n.a.	n.a.	0.07 ± 0.00	n.a.	n.d.	0.02 ± 0.00	n.a.
IP	0.02 ± 0.00	n.a.	n.a.	0.05 ± 0.00	n.a.	0.01 ± 0.00	0.01 ± 0.00	n.a.
tPAH	**58.6 ± 12.2**			**98.2 ± 12.5**	**n.a.**	**117 ± 6**	**44.3 ± 1.7**	
May								
N	n.d.	n.d.	n.d.	n.d.	n.d.	3.42 ± 0.80	n.d.	n.d.
Ace	n.d.	n.d.	n.d.	7.80 ± 1.05	n.d.	n.d.	n.d.	n.d.
Ac	5.51 ± 0.12	29.6 ± 3.4	11.9 ± 3.3	11.0 ± 0.1	8.29 ± 1.73	4.14 ± 1.01	35.3 ± 0.2	7.45 ± 0.37
F	4.04 ± 0.32	2.54 ± 0.18	2.03 ± 0.01	3.11 ± 0.51	1.87 ± 0.15	1.84 ± 0.04	n.d.	0.50 ± 0.10
P	7.18 ± 1.63	11.1 ± 0.9	9.51 ± 0.62	10.1 ± 0.8	8.45 ± 1.90	9.40 ± 2.61	13.6 ± 1.3	6.55 ± 0.63
A	0.97 ± 0.07	1.29 ± 0.22	0.62 ± 0.09	0.76 ± 0.16	0.77 ± 0.11	1.59 ± 0.29	1.18 ± 0.15	0.36 ± 0.05
Fluor	13.5 ± 3.6	18.0 ± 4.3	15.7 ± 2.3	14.2 ± 1.7	9.49 ± 1.37	26.0 ± 7.8	16.9 ± 2.7	6.82 ± 0.73
Py	8.46 ± 1.64	12.7 ± 2.7	17.8 ± 3.9	14.6 ± 1.0	7.19 ± 0.59	6.85 ± 1.89	46.5 ± 13.0	7.62 ± 0.68
BaA	42.9 ± 5.3	62.3 ± 10.1	61.5 ± 3.0	22.4 ± 1.1	22.3 ± 2.7	77.5 ± 8.8	80.1 ± 10.4	30.1 ± 5.9
Chr	24.7 ± 4.8	46.2 ± 7.2	33.5 ± 2.8	28.3 ± 2.2	9.37 ± 0.72	42.9 ± 4.1	38.9 ± 5.4	12.9 ± 3.1
BbF	12.1 ± 2.2	13.7 ± 0.5	6.71 ± 0.59	9.75 ± 0.86	4.74 ± 0.72	24.0 ± 7.0	15.6 ± 0.1	7.66 ± 0.74
BkF	6.91 ± 1.05	10.2 ± 2.1	8.97 ± 1.41	2.88 ± 0.34	2.15 ± 0.30	8.34 ± 1.8	8.66 ± 0.63	4.06 ± 0.74
BaP	n.d.	n.d.	8.46 ± 1.69	n.d.	1.45 ± 0.31	n.d.	n.d.	4.49 ± 0.23
DBahA	n.d.	9.82 ± 2.30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BghiP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
IP	n.d.	0.03 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
tPAH	**126 ± 15**	**218 ± 15**	**177 ± 3**	**125 ± 2**	**76.0 ± 3.3**	**206 ± 4**	**257 ± 17**	**88.5 ± 3.2**
June								
N	0.17 ± 0.04	n.a.	n.a.	n.d.	n.a.	0.16 ± 0.02	0.06 ± 0.01	n.a.
Ace	n.d.	n.a.	n.a.	n.d.	n.a.	3.12 ± 1.12	n.d.	n.a.
Ac	13.9 ± 1.1	n.a.	n.a.	25.0 ± 5.5	n.a.	13.6 ± 4.4	6.22 ± 1.30	n.a.
F	2.15 ± 0.45	n.a.	n.a.	3.07 ± 0.79	n.a.	1.99 ± 0.36	0.93 ± 0.20	n.a.
P	2.91 ± 0.57	n.a.	n.a.	5.60 ± 0.19	n.a.	5.79 ± 0.69	2.53 ± 0.72	n.a.
A	0.40 ± 0.04	n.a.	n.a.	0.72 ± 0.20	n.a.	0.57 ± 0.07	0.15 ± 0.05	n.a.
Fluor	4.71 ± 0.26	n.a.	n.a.	11.3 ± 2.5	n.a.	12.5 ± 1.4	4.08 ± 1.19	n.a.
Py	6.38 ± 1.36	n.a.	n.a.	10.9 ± 0.3	n.a.	9.35 ± 0.49	3.48 ± 0.39	n.a.
BaA	14.6 ± 2.1	n.a.	n.a.	39.7 ± 4.7	n.a.	33.0 ± 4.2	10.7 ± 0.8	n.a.
Chr	7.25 ± 1.11	n.a.	n.a.	16.4 ± 2.2	n.a.	19.2 ± 1.6	6.00 ± 0.76	n.a.
BbF	3.21 ± 0.50	n.a.	n.a.	n.d.	n.a.	n.d.	4.54 ± 0.24	n.a.
BkF	2.13 ± 0.48	n.a.	n.a.	n.d.	n.a.	n.d.	2.48 ± 0.08	n.a.
BaP	n.d.	n.a.	n.a.	6.05 ± 0.57	n.a.	n.d.	n.d.	n.a.
DBahA	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	1.16 ± 0.06	n.a.
BghiP	n.d.	n.a.	n.a.	n.d.	n.a.	0.10 ± 0.00	0.16 ± 0.01	n.a.
IP	0.01 ± 0.00	n.a.	n.a.	n.d.	n.a.	0.01 ± 0.00	0.02 ± 0.00	n.a.
tPAH	**57.8 ± 2.5**			**119 ± 10**		**99.3 ± 14.4**	**42.6 ± 2.3**	

Table A4 (Cont.)

PAHs \ Site	1	2	3	4	5	6	7	8
July								
N	4.10 ± 0.42	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
Ace	n.d.	n.a.	n.a.	n.d.	n.a.	n.d.	n.d.	n.a.
Ac	7.31 ± 0.85	n.a.	n.a.	43.2 ± 7.5	n.a.	49.4 ± 10.9	0.53 ± 0.03	n.a.
F	2.31 ± 0.16	n.a.	n.a.	7.32 ± 1.05	n.a.	6.23 ± 1.30	1.23 ± 0.08	n.a.
P	6.58 ± 0.30	n.a.	n.a.	6.10 ± 0.82	n.a.	8.19 ± 1.56	4.93 ± 1.96	n.a.
A	0.72 ± 0.05	n.a.	n.a.	1.24 ± 0.00	n.a.	0.98 ± 0.05	0.45 ± 0.03	n.a.
Fluor	4.99 ± 0.35	n.a.	n.a.	11.1 ± 0.1	n.a.	11.4 ± 1.3	5.33 ± 1.36	n.a.
Py	6.95 ± 0.62	n.a.	n.a.	6.40 ± 0.75	n.a.	7.20 ± 0.47	11.5 ± 2.0	n.a.
BaA	15.5 ± 1.1	n.a.	n.a.	22.7 ± 2.6	n.a.	33.4 ± 1.3	11.5 ± 0.2	n.a.
Chr	8.85 ± 0.64	n.a.	n.a.	13.6 ± 1.9	n.a.	15.3 ± 2.2	6.03 ± 0.56	n.a.
BbF	n.d.	n.a.	n.a.	8.81 ± 0.79	n.a.	6.29 ± 1.23	3.46 ± 0.71	n.a.
BkF	n.d.	n.a.	n.a.	2.90 ± 0.60	n.a.	4.54 ± 0.71	n.d.	n.a.
BaP	n.d.	n.a.	n.a.	1.73 ± 0.17	n.a.	n.d.	n.d.	n.a.
DBahA	n.d.	n.a.	n.a.	n.d.	n.a.	1.74 ± 0.17	n.d.	n.a.
BghiP	0.03 ± 0.00	n.a.	n.a.	0.10 ± 0.00	n.a.	0.44 ± 0.10	n.d.	n.a.
IP	0.04 ± 0.01	n.a.	n.a.	0.03 ± 0.00	n.a.	0.02 ± 0.00	0.02 ± 0.00	n.a.
tPAH	**57.3 ± 1.8**			**125 ± 2**		**145 ± 19**	**45.0 ± 3.8**	
August II								
N	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.
Ace	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.
Ac	7.44 ± 0.58	10.5 ± 0.7	3.21 ± 0.50	26.6 ± 1.3	2.00 ± 0.45	n.a.	9.06 ± 1.01	9.57 ± 0.27
F	1.70 ± 0.25	1.58 ± 0.28	0.58 ± 0.03	2.15 ± 0.18	0.53 ± 0.08	n.a.	2.56 ± 0.15	6.75 ± 0.98
P	9.74 ± 2.13	8.78 ± 0.52	8.15 ± 0.34	11.5 ± 0.6	9.23 ± 2.13	n.a.	9.79 ± 1.10	11.8 ± 1.5
A	0.31 ± 0.02	0.53 ± 0.15	0.57 ± 0.09	1.69 ± 0.28	0.54 ± 0.07	n.a.	0.89 ± 0.16	8.74 ± 1.91
Fluor	11.8 ± 0.6	16.8 ± 1.3	18.7 ± 2.0	30.6 ± 4.5	21.0 ± 3.4	n.a.	22.6 ± 1.5	36.3 ± 0.4
Py	18.0 ± 2.6	17.6 ± 1.5	22.7 ± 1.0	40.9 ± 7.3	24.7 ± 3.9	n.a.	25.1 ± 2.5	49.8 ± 14.2
BaA	76.3 ± 14.0	107 ± 13	72.8 ± 4.4	110 ± 9	83.5 ± 6.8	n.a.	105 ± 8	149 ± 16
Chr	97.9 ± 4.9	84.8 ± 6.6	90.4 ± 4.9	68.8 ± 6.2	104 ± 15	n.a.	66.3 ± 3.7	137 ± 15
BbF	32.7 ± 4.2	35.3 ± 1.4	35.1 ± 3.0	23.1 ± 2.1	32.8 ± 4.6	n.a.	17.6 ± 2.0	78.4 ± 9.4
BkF	47.5 ± 11.2	29.7 ± 1.7	44.9 ± 4.1	36.8 ± 3.3	43.0 ± 6.0	n.a.	29.4 ± 2.9	113 ± 9
BaP	41.0 ± 4.6	46.4 ± 3.6	27.3 ± 2.5	33.7 ± 2.5	33.9 ± 5.4	n.a.	23.3 ± 2.9	119 ± 12
DBahA	75.3 ± 21.2	52.1 ± 9.0	n.d.	n.d.	n.d.	n.a.	27.4 ± 3.4	312 ± 44
BghiP	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.d.	161 ± 6
IP	1.34 ± 0.28	1.10 ± 0.12	0.48 ± 0.05	n.d.	n.d.	n.a.	0.67 ± 0.12	n.d.
tPAH	**421 ± 52**	**413 ± 17**	**325 ± 9**	**386 ± 38**	**355 ± 30**		**340 ± 6**	**1191 ± 92**

n.d. – not detected; n.a. – data not available.

Table A5 – BSAF values of individual PAHs.

	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8
January								
Ac	56.5	89.4	59.4	61.2	4.79	4.07	2.50	3.31
F	18.5	37.5	128	17.9	32.3	20.3	27.9	55.2
P	28.1	47.2	84.3	141	26.7	56.0	41.5	183
A	13.5	351	39.6	43.0	10.1	61.9	21.8	71.8
Fluor	4.67	28.3	33.0	291	15.8	274	20.9	48.4
Py	8.92	51.5	28.8	18.4	17.5	94.6	29.1	53.6
BaA	90.8	83.6	39.9	656	131	109	249	487
Chr	132	592	177	—	502	123	184	394
BbF	75.5	—	32.3	558	47.1	62.4	—	151
BkF	—	—	132	742	—	—	—	452
BaP	8.64	—	55.1	—	—	—	—	—
DBahA	—	—	—	—	17.4	2.60	—	—
BghiP	—	1861	—	—	—	4.71	—	—
IP	—	6605	—	—	422	1.06	—	33.3
March								
Ac	1.45	—	—	—	—	1.86	—	—
F	1.16	—	—	—	—	2.69	—	—
P	0.50	—	—	97.4	—	29.8	21.1	—
A	5.15	—	—	77.0	—	9.49	18.5	—
Fluor	24.4	—	—	281	—	163	60.0	—
Py	22.7	—	—	18.8	—	62.1	31.2	—
BaA	47.8	—	—	486	—	225	138	—
Chr	33.9	—	—	246	—	310	296	—
BbF	26.8	—	—	—	—	—	—	—
BkF	22.0	—	—	—	—	—	—	—
BaP	—	—	—	—	—	—	—	—
DBahA	—	—	—	—	—	—	—	—
BghiP	—	—	—	—	—	—	—	—
IP	—	—	—	—	—	—	—	—
May								
Ac	0.98	13.1	17.8	3.06	—	1.07	58.0	3.13
F	3.85	5.77	11.5	5.04	—	3.17	—	1.74
P	13.4	40.9	35.4	23.4	—	11.0	83.0	31.9
A	18.6	16.5	17.0	7.87	—	11.4	52.5	7.70
Fluor	23.1	32.7	63.8	35.8	—	13.7	182	40.8
Py	18.8	31.2	74.0	86.3	—	8.95	492	56.0
BaA	134	157	253	50.7	—	55.1	658	139
Chr	167	214	258	117	—	96.2	441	61.3
BbF	139	104	55.9	—	—	—	399	—
BkF	—	—	—	—	—	—	1396	—
BaP	—	—	—	—	—	—	—	—
DBahA	—	—	—	—	—	—	—	—
BghiP	—	—	—	—	—	—	—	—
IP	—	—	—	—	—	—	—	—

Table A5 (Cont.)

	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8
June								
Ac	—	—	—	5.69	—	4.14	—	—
F	—	—	—	7.30	—	13.4	14.5	—
P	7.34	—	—	11.8	—	9.06	8.89	—
A	6.60	—	—	23.8	—	8.80	5.70	—
Fluor	11.8	—	—	18.0	—	60.7	25.2	—
Py	15.0	—	—	14.8	—	40.2	20.6	—
BaA	49.3	—	—	52.4	—	86.8	66.8	—
Chr	34.4	—	—	59.4	—	253	100	—
BbF	—	—	—	—	—	—	139	—
BkF	28.0	—	—	—	—	—	29.2	—
BaP	—	—	—	—	—	—	—	—
DBahA	—	—	—	—	—	—	—	—
BghiP	—	—	—	—	—	—	—	—
IP	—	—	—	—	—	—	—	—
July								
Ac	—	—	—	48.3	—	—	—	—
F	13.5	—	—	45.8	—	—	2.92	—
P	85.4	—	—	25.4	—	42.8	12.1	—
A	32.1	—	—	29.5	—	32.8	6.81	—
Fluor	66.0	—	—	67.8	—	87.6	41.7	—
Py	125	—	—	40.4	—	99.6	167	—
BaA	189	—	—	133	—	445	65.4	—
Chr	162	—	—	148	—	223	76.6	—
BbF	—	—	—	—	—	—	122	—
BkF	—	—	—	—	—	81.4	62.7	—
BaP	—	—	—	—	—	—	—	—
DBahA	—	—	—	—	—	—	—	—
BghiP	—	—	—	—	—	—	—	—
IP	—	—	—	—	—	—	—	—
August II								
Ac	—	42.3	—	—	—	—	—	—
F	11.2	9.14	1.69	—	3.26	—	—	14.1
P	86.4	39.4	22.4	16.6	39.1	—	31.1	24.8
A	13.7	19.8	15.5	11.1	10.0	—	19.0	127
Fluor	256	410	122	41.5	114	—	146	155
Py	467	231	305	155	174	—	295	311
BaA	1065	859	1420	588	1101	—	941	631
Chr	—	988	—	—	—	—	—	—
BbF	—	—	—	—	—	—	—	—
BkF	—	—	—	—	—	—	—	—
BaP	—	—	—	—	—	—	—	—
DBahA	—	—	—	—	—	—	—	—
BghiP	—	—	—	—	—	—	—	—
IP	—	—	—	—	—	—	—	—

Table A6 – Superoxide dismutase activity (U mg^{-1} prot) in the digestive gland of *Ruditapes decussatus.*

	Site	August I	October	November	December	January	March	May	June	July	August II
Mit SOD	1	2.90 ± 0.41	3.54 ± 0.83	20.9 ± 0.0	8.73 ± 0.88	4.50 ± 0.63	14.0 ± 1.2	11.0 ± 1.3	29.9 ± 0.0	3.40 ± 0.12	5.58 ± 1.62
	2	4.43 ± 0.00	3.94 ± 0.92	23.2 ± 2.3	5.87 ± 0.26	1.16 ± 0.00	n.a.	6.21 ± 0.72	n.a.	n.a.	13.7 ± 1.6
	3	n.a.	4.66 ± 0.81	9.95 ± 0.00	5.60 ± 0.23	8.30 ± 0.40	n.a.	3.03 ± 0.61	n.a.	n.a.	13.7 ± 1.0
	4	3.89 ± 0.00	7.32 ± 0.98	11.0 ± 0.5	1.58 ± 0.27	1.01 ± 0.00	3.50 ± 0.29	5.98 ± 0.55	21.3 ± 1.0	3.94 ± 0.48	22.9 ± 5.1
	5	7.33 ± 0.16	5.07 ± 0.27	9.79 ± 0.00	13.5 ± 0.6	6.34 ± 0.79	n.a.	9.04 ± 1.24	n.a.	n.a.	15.6 ± 1.4
	6	14.8 ± 0.8	13.8 ± 2.9	13.9 ± 2.6	6.22 ± 1.32	11.2 ± 0.3	13.1 ± 1.0	6.98 ± 1.80	32.2 ± 0.0	1.95 ± 0.23	n.a.
	7	10.3 ± 0.9	3.89 ± 0.35	8.39 ± 0.62	6.31 ± 0.47	4.34 ± 0.15	6.35 ± 0.42	7.51 ± 0.00	21.6 ± 1.7	8.08 ± 0.29	11.7 ± 1.7
	8	33.4 ± 2.5	3.77 ± 0.59	n.a.	18.0 ± 2.3	5.52 ± 0.00	n.a.	3.91 ± 0.15	n.a.	n.a.	16.8 ± 1.7
Cyt SOD	1	20.4 ± 0.8	38.9 ± 1.7	9.64 ± 0.46	63.0 ± 0.0	26.9 ± 1.2	32.3 ± 3.9	7.93 ± 1.42	30.1 ± 0.0	20.9 ± 0.0	5.71 ± 1.07
	2	23.9 ± 2.3	101 ± 7	19.4 ± 0.0	13.4 ± 1.1	28.1 ± 3.2	n.a.	5.91 ± 0.61	n.a.	n.a.	2.67 ± 1.53
	3	n.a.	41.6 ± 2.3	8.49 ± 0.45	14.4 ± 0.8	28.1 ± 1.2	n.a.	8.14 ± 0.00	n.a.	n.a.	2.75 ± 0.28
	4	21.6 ± 1.7	69.9 ± 13.9	11.4 ± 0.0	28.0 ± 2.1	18.7 ± 0.0	31.1 ± 4.2	8.25 ± 1.30	22.1 ± 0.8	20.9 ± 0.0	25.8 ± 1.1
	5	36.5 ± 2.7	97.0 ± 3.2	12.5 ± 0.7	9.03 ± 0.00	37.8 ± 2.3	n.a.	10.5 ± 1.2	n.a.	n.a.	16.6 ± 0.7
	6	10.3 ± 0.5	39.8 ± 0.0	25.6 ± 5.1	87.3 ± 7.9	29.2 ± 2.9	40.3 ± 4.8	6.52 ± 0.92	18.3 ± 1.3	12.0 ± 0.0	n.a.
	7	n.a.	51.6 ± 3.0	10.0 ± 1.0	58.5 ± 8.8	30.7 ± 3.1	72.7 ± 7.0	7.86 ± 0.00	30.0 ± 3.1	24.7 ± 1.7	22.6 ± 1.0
	8	27.1 ± 1.9	70.8 ± 8.0	n.a.	10.9 ± 1.1	24.7 ± 1.4	n.a.	3.39 ± 0.00	n.a.	n.a.	21.6 ± 0.9

n.a. – data not available.

Table A7 – Catalase activity (µmol min^{-1} mg^{-1} prot) in the digestive gland of *Ruditapes decussatus*.

Site	August I	October	November	December	January	March	May	June	July	Aug II
1	246 ± 10	219 ± 14	189 ± 12	434 ± 33	145 ± 6	305 ± 20	270 ± 13	307 ± 5	306 ± 5	110 ± 7
2	227 ± 12	305 ± 11	490 ± 48	383 ± 18	250 ± 9	n.a.	285 ± 10	n.a.	n.a.	108 ± 4
3	n.a.	251 ± 7	189 ± 11	250 ± 24	170 ± 9	n.a.	296 ± 15	n.a.	n.a.	70.2 ± 2.8
4	236 ± 5	269 ± 9	257 ± 12	247 ± 18	173 ± 7	318 ± 6	213 ± 7	340 ± 19	353 ± 50	220 ± 10
5	249 ± 5	325 ± 10	314 ± 22	292 ± 8	255 ± 17	n.a.	303 ± 13	n.a.	n.a.	140 ± 8
6	111 ± 2	242 ± 4	523 ± 21	617 ± 34	250 ± 13	577 ± 15	460 ± 7	317 ± 18	240 ± 13	n.a.
7	32.8 ± 1.1	368 ± 13	212 ± 20	285 ± 9	130 ± 3	522 ± 19	358 ± 17	432 ± 16	533 ± 18	211 ± 7
8	257 ± 13	323 ± 7	n.a.	269 ± 2	227 ± 4	n.a.	298 ± 59	n.a.	n.a.	233 ± 8

n.a. – data not available.

Table A8 – Total Glutathione Peroxidase (T GPx) and Se dependent Glutathione Peroxidase (Se GPx) activities (nmol min^{-1} mg^{-1} prot) in the digestive gland of *Ruditapes decussatus*.

		August I	October	November	December	January	March	May	June	July	August II
T GPx	Site 1	12.4 ± 1.5	16.2 ± 1.8	5.50 ± 1.45	5.12 ± 1.70	4.13 ± 0.52	14.3 ± 2.1	n.d.	42.1 ± 14.8	30.8 ± 12.9	7.05 ± 0.43
	Site 2	5.40 ± 1.11	4.21 ± 0.28	9.15 ± 0.97	10.8 ± 3.0	5.09 ± 1.01	n.a.	17.6 ± 2.8	n.a.	n.a.	2.06 ± 0.27
	Site 3	n.a.	7.91 ± 1.81	4.65 ± 0.99	9.19 ± 0.76	4.57 ± 0.73	n.a.	15.9 ± 3.8	n.a.	n.a.	4.59 ± 1.68
	Site 4	6.49 ± 1.13	12.3 ± 1.0	29.9 ± 0.5	1.63 ± 0.51	4.07 ± 0.84	10.7 ± 0.5	13.1 ± 3.9	23.8 ± 3.5	33.4 ± 12.5	11.0 ± 2.2
	Site 5	16.6 ± 2.2	19.2 ± 2.6	27.6 ± 0.3	13.0 ± 0.4	8.40 ± 1.64	n.a.	15.4 ± 0.2	n.a.	n.a.	7.29 ± 1.11
	Site 6	3.18 ± 0.31	3.65 ± 2.53	55.2 ± 1.9	5.63 ± 1.21	1.02 ± 0.56	14.1 ± 2.3	21.8 ± 3.7	16.6 ± 1.3	7.67 ± 6.64	n.a.
	Site 7	n.d.	14.9 ± 2.7	4.73 ± 0.42	6.36 ± 1.99	n.d.	12.7 ± 1.7	17.1 ± 3.2	28.0 ± 1.9	20.5 ± 1.9	11.3 ± 0.4
	Site 8	12.3 ± 1.1	5.08 ± 0.39	n.a.	5.79 ± 0.00	n.d.	n.a.	22.1 ± 1.4	n.a.	n.a.	11.0 ± 0.7
Se GPx	Site 1	0.86 ± 0.45	3.26 ± 0.60	2.71 ± 0.34	n.d.	n.d.	5.14 ± 0.38	3.79 ± 0.34	43.6 ± 3.6	45.0 ± 12.9	3.40 ± 0.12
	Site 2	0.91 ± 0.42	2.59 ± 0.92	4.57 ± 1.60	3.70 ± 0.75	n.d.	n.a.	4.29 ± 0.00	n.a.	n.a.	4.93 ± 1.16
	Site 3	n.a.	3.49 ± 0.39	3.47 ± 1.22	3.27 ± 0.15	n.d.	n.a.	4.32 ± 0.00	n.a.	n.a.	2.39 ± 0.19
	Site 4	1.44 ± 0.31	4.51 ± 0.72	0.63 ± 0.47	0.42 ± 0.11	n.d.	3.70 ± 0.37	4.18 ± 0.00	31.5 ± 3.4	43.3 ± 8.0	n.d.
	Site 5	n.d.	6.64 ± 0.80	0.97 ± 0.57	3.24 ± 0.60	n.d.	n.a.	n.d.	n.a.	n.a.	n.d.
	Site 6	0.60 ± 0.00	2.92 ± 0.83	3.63 ± 2.04	2.11 ± 0.00	8.28 ± 0.56	5.34 ± 1.38	n.d.	29.4 ± 4.6	38.5 ± 13.7	n.a.
	Site 7	n.a.	6.45 ± 0.62	3.71 ± 0.32	2.00 ± 0.56	2.24 ± 0.36	3.36 ± 0.58	n.d.	42.3 ± 3.6	27.2 ± 19.1	22.4 ± 1.4
	Site 8	2.40 ± 0.36	4.08 ± 0.11	n.a.	3.78 ± 0.63	1.69 ± 0.23	n.a.	n.d.	n.a.	n.a.	8.04 ± 1.81

n.a. – data not available; n.d. – not detected.

Table A9 – Spearman by Ranks Correlation coefficients between antioxidant enzymes activity and lipid peroxidation in the digestive gland of *Ruditapes decussatus* and PAHs content in the whole soft tissues of clams. Significant coefficients are marked with *.

	Mit SOD	Cyt SOD	CAT	T GPx	Se GPx	LPO
N	0.0709	-0.0357	0.1017	0.1266	0.0540	-0.0080
Ace	-0.2303	0.3049*	0.1346	-0.0635	0.0223	0.2006
Ac	-0.2575*	0.0131	-0.1782	-0.0256	0.0417	-0.2223
F	-0.1860	0.0045	-0.4031*	-0.1172	-0.0247	-0.4190*
P	-0.1107	-0.1649	-0.4721*	-0.2878*	-0.2693*	-0.5173*
A	-0.1699	0.0546	-0.3932*	-0.3036*	-0.2380	-0.4079*
Fluo	0.0134	-0.2010	-0.4520*	-0.3897*	-0.2344	-0.3825*
Py	0.0043	-0.3303*	-0.4274*	-0.3525*	-0.2035	-0.4651*
BaA	-0.0735	-0.2500*	-0.5046*	-0.4154*	-0.2489*	-0.4810*
Ch	-0.1631	-0.1550	-0.3300*	-0.3854*	-0.1935	-0.1470
BbF	0.0144	-0.1117	-0.2731*	-0.0747	-0.0873	-0.0987
BkF	-0.0064	0.0948	-0.0594	-0.1061	0.3111*	-0.3164*
BaP	-0.1818	0.2467*	0.0063	-0.0594	0.3205*	0.0337
DBA	-0.2023	0.0208	-0.1645	-0.2561*	0.0596	-0.1999
BPer	-0.2175	0.2420	0.0538	-0.0841	0.2761*	0.0337
IP	-0.2376	0.1276	-0.1590	-0.2779*	0.1023	-0.2019
tPAH	-0.1064	-0.1408	-0.5601*	-0.4172*	-0.2553*	-0.4436*
2+3 rings	0.0449	-0.1330	-0.4827*	-0.4044*	-0.2734*	-0.4187*
4 rings	-0.0197	-0.2560*	-0.1656	-0.1480	-0.0831	-0.0828
5+6 rings	-0.1368	-0.1240	-0.4052*	-0.4222*	-0.2251	-0.2779

Table A10 – Polycyclic aromatic hydrocarbons (PAH) concentrations (mean±standard deviation) (ng g^{-1} w.w.), in the edible part of clams at site 5 and transplanted to site 7.

	Days	0	1	3	7	14	28
Site 5	N	n.d.	n.d.	n.d.	1.49 ± 0.06	14.5 ± 4.3	n.d.
	Ace	n.d.	9.06 ± 2.65	n.d.	n.d.	n.d.	n.d.
	Ac	54.9 ± 8.1	118 ± 13	78.1 ± 0.5	57.5 ± 7.0	91.0 ± 26.5	169 ± 29
	F	5.01 ± 0.55	6.05 ± 0.50	5.80 ± 1.40	14.2 ± 1.9	24.7 ± 5.8	19.4 ± 3.5
	P	6.47 ± 1.00	19.0 ± 3.5	18.2 ± 3.9	32.9 ± 3.8	28.1 ± 3.6	15.6 ± 1.6
	A	0.81 ± 0.20	2.47 ± 0.57	2.42 ± 0.26	8.37 ± 1.58	2.85 ± 0.57	1.53 ± 0.25
	Fluor	16.1 ± 4.8	44.1 ± 8.2	39.3 ± 5.6	72.0 ± 17.3	49.2 ± 10.6	28.4 ± 2.5
	Py	25.3 ± 5.9	30.9 ± 2.2	20.5 ± 5.5	97.0 ± 16.8	70.1 ± 7.7	56.5 ± 3.9
	BaA	29.9 ± 7.1	88.5 ± 6.1	70.7 ± 5.8	70.9 ± 10.8	65.9 ± 4.5	92.7 ± 4.1
	Ch	16.2 ± 2.9	52.1 ± 4.8	41.5 ± 3.6	76.8 ± 16.5	36.4 ± 7.5	35.2 ± 2.1
	BbF	6.22 ± 0.49	30.3 ± 4.0	25.4 ± 5.0	17.6 ± 1.7	13.5 ± 1.2	23.7 ± 1.7
	BkF	13.1 ± 0.8	4.42 ± 1.67	9.59 ± 2.15	1.76 ± 0.36	17.4 ± 3.2	n.d.
	BaP	n.d.	n.d.	n.d.	0.07 ± 0.02	n.d.	n.d.
	DBA	0.25 ± 0.06	n.d.	n.d.	n.d.	n.d.	0.60 ± 0.08
	Bper	n.d.	0.06 ± 0.00	n.d.	n.d.	n.d.	0.06 ± 0.01
	IP	n.d.	n.d.	0.16 ± 0.00	n.d.	n.d.	0.03 ± 0.01
	2+3 rings	67.2 ± 8.2	154 ± 14	104 ± 4	114 ± 8	161 ± 28	205 ± 29
	4 rings	87.6 ± 10.8	216 ± 11	172 ± 10	317 ± 31	222 ± 16	213 ± 7
	5+6 rings	19.6 ± 0.9	34.8 ± 4.3	35.1 ± 5.5	19.4 ± 1.7	30.9 ± 3.4	24.4 ± 1.7
	tPAH	174 ± 14	405 ± 18	312 ± 12	451 ± 32	414 ± 32	442 ± 30
Transplanted from site 5 to site 7	N		n.d.	n.d.	n.d.	n.d.	n.d.
	Ace		n.d.	n.d.	n.d.	n.d.	n.d.
	Ac		304 ± 28	235 ± 73	136 ± 0	115 ± 45	109 ± 14
	F		8.12 ± 0.95	13.2 ± 0.9	18.6 ± 6.3	14.8 ± 4.1	8.31 ± 0.55
	P		27.5 ± 5.3	22.1 ± 1.0	38.6 ± 4.6	24.1 ± 3.3	17.0 ± 2.2
	A		3.38 ± 0.72	3.17 ± 0.21	9.55 ± 0.88	3.46 ± 0.61	1.12 ± 0.02
	Fluor		32.5 ± 6.0	37.0 ± 0.4	60.2 ± 10.6	31.1 ± 4.3	27.5 ± 2.9
	Py		82.9 ± 13.3	39.5 ± 3.0	71.2 ± 1.7	58.8 ± 6.9	40.1 ± 5.2
	BaA		97.3 ± 4.1	69.8 ± 6.7	42.6 ± 0.1	59.3 ± 13.1	45.1 ± 4.3
	Ch		37.4 ± 1.3	41.0 ± 4.1	24.4 ± 0.9	52.0 ± 4.5	38.7 ± 1.6
	BbF		n.d.	22.0 ± 5.3	22.6 ± 8.7	7.66 ± 2.19	12.4 ± 0.6
	BkF		n.d.	11.3 ± 1.8	5.19 ± 1.70	n.d.	4.98 ± 1.07
	BaP		1.66 ± 0.07	n.d.	n.d.	n.d.	n.d.
	DBA		n.d.	n.d.	n.d.	n.d.	n.d.
	Bper		n.d.	n.d.	n.d.	n.d.	n.d.
	IP		n.d.	n.d.	n.d.	n.d.	n.d.
	2+3 rings		343 ± 29	274 ± 73	203 ± 8	157 ± 45	135 ± 14
	4 rings		250 ± 15	187 ± 8	199 ± 11	201 ± 16	151 ± 8
	5+6 rings		1.66 ± 0.07	33.3 ± 5.6	27.8 ± 8.9	7.66 ± 2.19	17.4 ± 1.2
	tPAH		594 ± 32	495 ± 73	429 ± 16	366 ± 48	304 ± 16

n.d. – not detected.

Table A11 – Polycyclic aromatic hydrocarbons (PAH) concentrations (mean±standard deviation) (ng g^{-1} w.w.), in the edible part of clams remaining at site 5 and backtransplanted from site 7 to site 5.

	Days	28	35	42	50	56
Site 5	N	n.d.	n.d.	n.d.	n.d.	n.d.
	Ace	n.d.	n.d.	n.d.	n.d.	n.d.
	Ac	169 ± 29	80.2 ± 4.8	55.8 ± 1.6	63.4 ± 3.1	44.8 ± 2.4
	F	19.4 ± 3.5	7.27 ± 1.88	3.56 ± 0.60	6.20 ± 0.08	4.08 ± 0.16
	P	15.6 ± 1.6	15.9 ± 2.8	14.3 ± 2.2	16.6 ± 0.3	13.3 ± 0.4
	A	1.53 ± 0.25	2.34 ± 0.44	1.30 ± 0.07	1.66 ± 0.03	1.73 ± 0.09
	Fluor	28.4 ± 2.5	39.0 ± 5.9	41.9 ± 5.3	45.3 ± 4.1	33.6 ± 0.7
	Py	56.5 ± 3.9	24.1 ± 1.6	30.2 ± 3.0	35.9 ± 2.0	24.3 ± 2.3
	BaA	92.7 ± 4.1	65.5 ± 12.2	89.3 ± 4.4	111 ± 2	66.6 ± 0.6
	Ch	35.2 ± 2.1	41.1 ± 9.3	53.1 ± 3.9	62.9 ± 1.0	39.9 ± 0.5
	BbF	23.7 ± 1.7	23.7 ± 6.4	20.1 ± 5.9	19.6 ± 0.7	12.4 ± 0.2
	BkF	n.d.	7.85 ± 1.06	11.9 ± 1.1	13.5 ± 0.4	n.d.
	BaP	n.d.	n.d.	n.d.	4.33 ± 0.52	2.18 ± 0.20
	DBA	0.60 ± 0.08	n.d.	1.59 ± 0.56	n.d.	n.d.
	Bper	0.06 ± 0.01	n.d.	n.d.	n.d.	n.d.
	IP	0.03 ± 0.01	0.10 ± 0.03	0.15 ± 0.01	n.d.	0.02 ± 0.00
	2+3 rings	205 ± 29	106 ± 6	75.0 ± 2.8	87.8 ± 3.2	63.9 ± 8.2
	4 rings	213 ± 7	170 ± 17	215 ± 8	255 ± 5	164 ± 2
	5+6 rings	24.4 ± 1.7	31.7 ± 6.5	33.8 ± 6	37.4 ± 0.9	14.5 ± 0.3
	tPAH	442 ± 30	307 ± 19	323 ± 11	381 ± 6	243 ± 3
Backtransplanted from site 7 to site 5	N	n.d.	n.d.	0.38 ± 0.10	0.86 ± 0.03	n.d.
	Ace	n.d.	n.d.	n.d.	n.d.	n.d.
	Ac	109 ± 14	113 ± 0	201 ± 31	161 ± 21	84.9 ± 10.4
	F	8.31 ± 0.55	23.3 ± 2.3	10.7 ± 0.4	8.58 ± 0.62	5.89 ± 1.03
	P	17.0 ± 2.2	27.4 ± 5.4	19.8 ± 5.3	23.9 ± 0.1	13.5 ± 2.2
	A	1.12 ± 0.02	5.90 ± 1.30	2.56 ± 0.59	2.26 ± 0.03	1.26 ± 0.07
	Fluor	27.5 ± 2.9	47.2 ± 7.6	39.1 ± 9.7	52.9 ± 1.1	35.9 ± 4.4
	Py	40.1 ± 5.2	45.0 ± 7.5	40.6 ± 3.7	33.9 ± 1.5	28.6 ± 3.8
	BaA	45.1 ± 4.3	84.7 ± 10.7	76.0 ± 17.2	87.0 ± 0.9	77.5 ± 12.0
	Ch	38.7 ± 1.6	48.6 ± 4.4	44.9 ± 10.0	57.0 ± 0.0	46.8 ± 6.2
	BbF	12.4 ± 0.6	12.2 ± 3.3	33.0 ± 2.4	9.41 ± 1.20	10.1 ± 2.3
	BkF	4.98 ± 1.07	12.3 ± 1.7	4.98 ± 1.24	9.78 ± 0.99	10.2 ± 1.1
	BaP	n.d.	n.d.	2.16 ± 0.17	1.87 ± 0.22	3.06 ± 0.03
	DBA	n.d.	n.d.	2.89 ± 0.68	1.93 ± 0.43	n.d.
	Bper	n.d.	n.d.	0.15 ± 0.05	1.08 ± 0.22	1.52 ± 0.22
	IP	n.d.	n.d.	n.d.	n.d.	0.34 ± 0.01
	2+3 rings	135 ± 14	170 ± 6	234 ± 31	196 ± 21	106 ± 11
	4 rings	151 ± 8	226 ± 16	201 ± 22	231 ± 2	189 ± 15
	5+6 rings	17.4 ± 1.2	24.6 ± 3.7	43.1 ± 2.8	24.1 ± 1.6	25.3 ± 2.5
	tPAH	304 ± 16	420 ± 17	478 ± 39	451 ± 21	320 ± 18

n.d. – not detected.

Table A12 – Antioxidant enzyme activities (mean±SD), in the gills and digestive gland of clams from site 5 and transplanted to site 7. Values significantly different from day 0 are marked with * (p<0.05).

	Days	Site 5		Transplanted to site 7	
		Gills	DG	Gills	DG
Mit SOD (U mg^{-1} prot)	0	8.75 ± 2.36	3.61 ± 0.48	8.75 ± 2.36	3.61 ± 0.48
	1	9.37 ± 1.18	3.79 ± 0.80	7.42 ± 1.30	3.87 ± 0.38
	3	5.05 ± 0.69*	4.73 ± 0.89*	4.75 ± 1.30*	4.80 ± 1.06*
	7	5.69 ± 1.08*	3.67 ± 0.80	5.25 ± 1.13*	3.76 ± 0.79
	14	12.0 ± 1.8*	2.33 ± 0.19*	17.5 ± 3.8*	3.02 ± 0.70
	28	11.7 ± 2.3	5.06 ± 1.10*	13.7 ± 2.9*	4.12 ± 0.96
Cyt SOD (U mg^{-1} prot)	0	116 ± 7	34.1 ± 4.6	116 ± 7	34.1 ± 4.6
	1	114 ± 15	33.0 ± 3.9	124 ± 18	25.1 ± 1.8*
	3	76.1 ± 13.6*	25.9 ± 2.8*	80.7 ± 11.9*	37.2 ± 3.4
	7	80.9 ± 14.1*	19.1 ± 8.4*	83.2 ± 11.3*	24.8 ± 2.9*
	14	68.8 ± 9.2	37.3 ± 4.7	55.9 ± 9.7*	30.9 ± 3.4
	28	83.3 ± 12.6*	40.7 ± 2.5*	89.2 ± 20.7	40.7 ± 3.6*
CAT (µmol min^{-1} mg^{-1} prot)	0	287 ± 25	242 ± 15	287 ± 25	242 ± 15
	1	374 ± 24*	273 ± 44	305 ± 44	255 ± 9
	3	276 ± 20	262 ± 39	303 ± 35	247 ± 44
	7	291 ± 49	249 ± 37	324 ± 47	216 ± 15
	14	344 ± 40	187 ± 24*	292 ± 33	195 ± 33*
	28	420 ± 40*	144 ± 21*	326 ± 27	152 ± 14*
TGPx (nmol min^{-1} mg^{-1} prot)	0	28.2 ± 7.0	15.4 ± 3.0	28.2 ± 7.0	15.4 ± 3.0
	1	35.2 ± 4.4	17.6 ± 2.1	28.5 ± 4.2	31.3 ± 5.7*
	3	17.8 ± 2.5*	21.4 ± 3.4*	11.5 ± 1.6*	15.6 ± 2.0
	7	19.5 ± 2.1*	20.6 ± 1.8*	19.0 ± 3.9*	22.1 ± 3.4*
	14	12.9 ± 1.8*	27.5 ± 4.1*	11.1 ± 1.3*	22.3 ± 2.5*
	28	21.7 ± 3.6*	28.2 ± 3.5*	20.4 ± 1.9*	30.4 ± 3.1*
Se GPx (nmol min^{-1} mg^{-1} prot)	0	13.0 ± 1.9	9.61 ± 1.08	13.0 ± 1.9	9.61 ± 1.08
	1	18.7 ± 2.0*	10.5 ± 1.2	14.3 ± 2.5	10.4 ± 1.1
	3	7.26 ± 1.85*	8.08 ± 1.17*	5.77 ± 1.35*	8.66 ± 1.13*
	7	9.76 ± 2.24*	8.12 ± 1.74	6.37 ± 1.40*	9.04 ± 0.45
	14	4.90 ± 1.00*	11.7 ± 2.9	5.20 ± 0.91*	10.8 ± 1.6
	28	6.93 ± 1.61*	12.7 ± 2.5*	11.7 ± 2.2	12.9 ± 2.5*

Table A13 – BPH and GST activities and LPO (mean±SD), in the gills and digestive gland of clams from site 5 and transplanted to site 7. Values significantly different from day 0 are marked with * (p<0.05).

	Days	Site 5		Transplanted to site 7	
		Gills	DG	Gills	DG
BPH (f.u. $min^{-1}mg^{-1}$ prot)	0	n.d.	1.17 ± 0.32	n.d.	1.17 ± 0.32
	1		0.83 ± 0.34		4.19 ± 0.67*
	3		0.54 ± 0.08*		1.75 ± 0.46
	7		0.61 ± 0.10*		0.38 ± 0.03*
	14		0.21 ± 0.05*		0.16 ± 0.00*
	28		0.14 ± 0.02*		0.32 ± 0.04*
GST (nmol min^{-1} mg^{-1} prot)	0	889 ± 64	424 ± 39	889 ± 64	424 ± 39
	1	1570 ± 160*	447 ± 64	1663 ± 327*	548 ± 25*
	3	911 ± 142	502 ± 59*	923 ± 151	328 ± 66*
	7	954 ± 135	373 ± 36*	885 ± 110	363 ± 60
	14	1065 ± 192	492 ± 75*	892 ± 92	304 ± 38*
	28	1690 ± 354*	401 ± 68	1549 ± 345*	520 ± 35*
MDA + 4-HNE (nmol g^{-1} prot)	0	592 ± 70	714 ± 143	592 ± 70	714 ± 143
	1	658 ± 109	1444 ± 175*	613 ± 74	1049 ± 128*
	3	855 ± 97*	1211 ± 119*	760 ± 133	1376 ± 127*
	7	912 ± 158*	1280 ± 107*	726 ± 47	1839 ± 115*
	14	839 ± 106*	1031 ± 124*	1061 ± 183*	2247 ± 261*
	28	705 ± 184	1029 ± 182*	854 ± 142*	1009 ± 102*

n.d. - not detected

Table A14 – Antioxidant enzyme activities (mean±SD), in the gills and digestive gland of clams from site 5 and backtransplanted from site 7 to site 5.

	Days	Site 5		Backtransplanted	
		Gills	DG	Gills	DG
Mit SOD (U mg^{-1} prot)	28	11.7 ± 2.3	5.06 ± 1.10	13.7 ± 2.9	4.12 ± 0.96
	35	10.1 ± 2.4	3.91 ± 0.82	8.08 ± 1.99	3.08 ± 0.69
	42	11.0 ± 2.4	2.79 ± 0.37	11.6 ± 3.0	4.37 ± 0.67
	50	20.9 ± 3.7	5.91 ± 1.08	9.74 ± 1.72	4.74 ± 0.31
	56	30.9 ± 10.1	5.93 ± 1.08	12.6 ± 3.8	5.91 ± 1.11
Cyt SOD (U mg^{-1} prot)	28	83.3 ± 12.6	40.7 ± 2.5	89.2 ± 20.7	40.7 ± 3.6
	35	73.5 ± 5.2	41.0 ± 3.3	57.4 ± 6.6	40.9 ± 2.3
	42	68.8 ± 7.3	41.1 ± 2.8	59.0 ± 6.4	42.4 ± 1.7
	50	68.9 ± 8.3	38.3 ± 3.5	62.4 ± 12.0	47.3 ± 3.3
	56	71.5 ± 10.3	41.6 ± 4.5	71.0 ± 12.1	42.1 ± 3.7
CAT (µmol min^{-1} mg^{-1} prot)	28	420 ± 40	144 ± 21	326 ± 27	152 ± 14
	35	408 ± 37	158 ± 30	382 ± 40	172 ± 8
	42	457 ± 43	130 ± 12	364 ± 28	168 ± 26
	50	345 ± 49	126 ± 16	347 ± 66	136 ± 4
	56	461 ± 43	160 ± 33	337 ± 29	123 ± 15
TGPx (nmol min^{-1} mg^{-1} prot)	28	21.7 ± 3.6	28.2 ± 3.5	20.4 ± 1.9	30.4 ± 3.1
	35	16.1 ± 2.2	29.1 ± 4.9	10.4 ± 2.0	30.6 ± 4.3
	42	12.3 ± 1.7	27.6 ± 1.9	11.8 ± 1.2	28.7 ± 2.3
	50	13.6 ± 2.2	28.1 ± 2.5	12.6 ± 2.0	28.7 ± 3.3
	56	12.6 ± 2.1	28.2 ± 2.7	17.8 ± 2.1	32.0 ± 2.8
Se GPx (nmol min^{-1} mg^{-1} prot)	28	6.93 ± 1.61	12.7 ± 2.5	11.7 ± 2.2	12.9 ± 2.5
	35	10.5 ± 1.7	15.5 ± 3.0	7.18 ± 1.44	13.6 ± 2.0
	42	8.48 ± 1.47	13.0 ± 1.9	7.76 ± 1.14	14.2 ± 2.2
	50	6.15 ± 1.15	14.6 ± 1.8	5.93 ± 1.07	13.9 ± 2.0
	56	6.15 ± 0.92	12.5 ± 1.3	7.91 ± 1.04	14.4 ± 1.4

Table A15 – BPH and GST activities and LPO (mean±SD), in the gills and digestive gland of clams from site 5 and backtransplanted from site 7 to site 5.

	Days	Site 5		Backtransplanted	
		Gills	DG	Gills	DG
BPH (f.u. $min^{-1}mg^{-1}$ prot)	28	n.d.	0.14 ± 0.02	n.d.	0.32 ± 0.04
	35		0.38 ± 0.17		0.45 ± 0.15
	42		0.27 ± 0.08		0.39 ± 0.05
	50		0.85 ± 0.25		2.07 ± 0.57
	56		0.85 ± 0.30		1.47 ± 0.07
GST (nmol min^{-1} mg^{-1} prot)	28	1690 ± 354	401 ± 68	1549 ± 345	520 ± 35
	35	1413 ± 140	549 ± 90	974 ± 96	580 ± 40
	42	1402 ± 66	406 ± 48	1011 ± 182	463 ± 54
	50	1282 ± 187	392 ± 49	954 ± 209	581 ± 81
	56	761 ± 104	369 ± 55	1035 ± 229	455 ± 58
MDA + 4-HNE (nmol g^{-1} prot)	28	705 ± 184	1029 ± 182	854 ± 142	1009 ± 102
	35	1038 ± 59	1313 ± 134	703 ± 78	873 ± 69
	42	1004 ± 130	1155 ± 80	1039 ± 52	1235 ± 203
	50	1069 ± 191	1070 ± 228	1122 ± 62	1198 ± 340
	56	1429 ± 289	973 ± 133	1257 ± 188	1031 ± 18

n.d. - not detected