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Polycyclic Aromatic Hydrocarbon Accumulation and Biomarker Responses in Cockles from Moroccan Mediterranean Coasts

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Abstract: This study aims to evaluate levels and effects of Polycyclic Aromatic Hydrocarbons (PAHs) in cockles (*Acanthocardia tuberculatae*) collected from two differently influenced areas in the Mediterranean Western Moroccan coasts. PAHs accumulation was studied in soft tissues using Soxhlet extraction and separation on silica column methods. The measure of those organic compounds was realized by Gas chromatography coupled to Mass spectrometer technics (GC/MS). The impact evaluation was carried out by the study of biochemical responses in gills and digestive gland using two enzymes activities: Glutathione S-Transferase (GST) and Acetylcholinesterase (AchE). The PAHs displayed significant rates of accumulation ranging from 10.12µg/g Dried Weight (DW) to 11.65µg/g (DW) respectively in Oued Laou and Martil sites. Pyrolytic and petrogenic origins were observed in both sites. Pyrolytic origin of PAHs was strongly detected in Oued Laou site while petrogenic origin was mostly detected in Martil site. Biochemical study revealed significant enzymatic response of GST and AchE in gills and digestive gland. The study showed significant biochemical response more important in Martil site than Oued Laou site traduced by GST induction and AchE inhibition. Those results seemed to be related to accumulation rates of PAHs, which was also suggested by the statistical analysis PCA.

Keywords: PAHs, *Acanthocardia Tuberculatae*, GST, AchE, Mediterranean Western Moroccan Coasts

Introduction

The Moroccan Mediterranean coastline is well known for its important socio-economical contribution to national economy. Thanks to its geographical and strategic position at the opening of the Mediterranean Sea over the Atlantic Ocean, it knows an increasing fishing, tourism and industry activities. However, this situation leads to serious threats of environment stability, especially on the western zone. Indeed, this latter is hardly exposed to an anthropogenic pressure resulting from urban and industrial discharges emanating from coastal agglomerations, harbors activities, fishing activities and maritime traffic through the strait of Gibraltar. Consequently, those activities conduct to important inputs of various pollutants into coastal area.

Among those pollutants Polycyclic Aromatic Hydrocarbons (PAHs) were considered as the most hazardous compounds. They were particularly used on marine-environment programs such as “Mussel Watch” in the USA and the French National Network (RNO) (Farrington *et al*., 1983; Beliaeff *et al*., 1997; Claisse *et al*., 1992). Moreover, particular attention was given to those organic micropollutants because of their potential toxicity, carcinogenic and mutagenic potency (Neff, 1979 ; Long *et al*., 1995; Azdi *et al*., 2006; Otávio *et al*., 2010; Er-Raioui *et al*., 2012). Furthermore, Environmental Protection Agency of United States (US-EPA) has designated 16 parental compounds as priority pollutants.

The use of sentinel organisms in PAHs assessment in marine environment constitute an interesting choice since the study of chemical accumulations in tissues might be extended to the study of biological responses

(Depledge and Fossi, 1994; Fossi *et al*., 2000 ; Bebianno and Barreira 2009; Francioni *et al*., 2010). Many marine organisms accumulate PAHs at concentrations well above those found in the surrounding environment (Andral *et al*., 2004; Bachelot, 2010) especially bivalves (RNO, 2003). They are mainly sedentary, filter-feeders and characterized by their high ability to bioaccumulate chemical substances dissolved in water or adsorbed to suspended particles (Solé *et al*., 2007; León *et al*., 2013). In addition, they could be excellent indicators of local inputs of pollution. Besides, bivalves have a low level of enzyme activity systems capable of metabolizing persistent organic pollutants such as aromatic hydrocarbons (Yap *et al*., 2008 ; León *et al*., 2013).

In this work, cockles (*Acanthocardia tuberculatae*) had been chosen for this study. They are bivalve species widely distributed along the western coasts of the Moroccan Mediterranean see. They live within the upper few centimeters of sediment. In addition, previous works showed that *A. tuberculatae* is an excellent indicator of contamination (Er-Raioui *et al*., 2009 ; Bouzid *et al*., 2011).

Most works carried out on Morocco's western Mediterranean coasts concern mainly monitoring studies of aquatic environment quality, detailing the physico-chemical and geochemical characterization of urban discharges and marine pollution, particularly by total hydrocarbons, metallic trace elements and harmful efflorescence and biotoxins (Er-Raioui *et al*., 2009; Bouzid *et al*., 2012; Omar *et al*., 2015; Khannous *et al*., 2013; Leblad *et al*., 2013). However, the effects of PAH accumulation in living organisms, have still not been studied in this area. In this research field, biomarkers are widely used in the monitoring of biological effects of contaminants in the marine environment (e.g. ICES, 2011).

Generally, biomarker concept refers to biochemical, physiological and histological changes in organisms that could be used to estimate either exposure to contaminants or the effects of pollution. Moreover, many studies have reported changes in these biomarkers associated with bivalve exposure to hydrocarbons (Baumard *et al*., 1999; Richardson *et al*., 2008; Bertrand *et al*., 2013; Nahrgang *et al*., 2013; Vidal-Liñán *et al*., 2010).

Once PAHs absorbed by bivalves, they are immediately metabolized by biotransformation enzymes. These transformation processes are usually classified into two phases: phase I and phase II reactions. The most studied enzyme involved in this latter we could mention the glutathione S transferase enzyme (GST). Indeed, it operates in the second stage of xenobiotic metabolism (phase II) and is considered as a defense biomarker (Sinaei *et al*., 2012).

On another hand, Acetylcholinesterase activity (AChE) is considered as biomarker of behavioral impact of neurotoxicity (Amiard-Triquet, 2009). Indeed, this impact is manifested by a malfunctioning of the neuromuscular system (Amiard *et al*., 2012; Cajaraville *et al*., 2000). In fact, AchE inhibition leads to the accumulation of acetylcholine in the synaptic space and keeps making a permanent transmission of nerve impulse, which usually leads to muscle tetany and organism death (Matozzo *et al*., 2006).

In this study the response of GST and AchE was carried out in tow organs: Gills and digestive glands. They are considered as the major target organs for pollution studies in bivalves (Lima *et al*., 2007; Vidal-Liñán *et al*., 2009; Lüchmann *et al*., 2011; Liu *et al*., 2012; Vidal-Liñán *et al*., 2015).

Digestive gland corresponds to the site of biotransformation activities (Livingstone, 1998) and is the main organ responsible of organic compounds metabolism, therefore, levels of biomarkers response to stress are considerably high.

On another hand, gills are located in external mantle cavity of the organism. They can directly interact with marine environment, therefore levels of oxidative processes are maximum in this tissue (Vidal-Liñán *et al*., 2015).

Consequently, this present work integrates for the first time on this area, both chemical and biochemical analysis. The research concerns, first, the study of PAHs bioaccumulation in cockles from Moroccan Mediterranean coasts, then it interests the evaluation of two biomarkers, one for exposure (GST) and the other for effect/damage (AchE) in two different organs, gills and digestive glands.

Materials and Methods

Sampling Sites

The area study is located in coastal fringe of the western Moroccan Mediterranean Sea. Sampling sites correspond to Martil and Oued laou beaches next to rivers mouths. Those sites were chosen because of the nature of pollution they are exposed to. Martil is characterized by industrial and domestic discharges emanating from Tetouan city and neighboring villages via Martil river. Oued laou receives less inputs compared to Martil site, it is mainly influenced by agricol activities. Moreover, this area is subjected to organic inputs of petrogenic hydrocarbons resulting from harbors activities (two harbors for pleasance and one for fisheries) (Fig. 1).

Experimental Design

Two hundred specimens of cockles (*A.Tuberculatae),* naturally present in marine environment, had been collected from each sampling sites, during the spring season (May 2012). They were transported to laboratory at +4°C in a cold box. Upon arrival to the laboratory, individuals were acclimated 24 h in aquariums in order to reduce the effects of transport stress. Subsequently, cockles were processed differently according to various analyses (chemical and biochemical analysis).

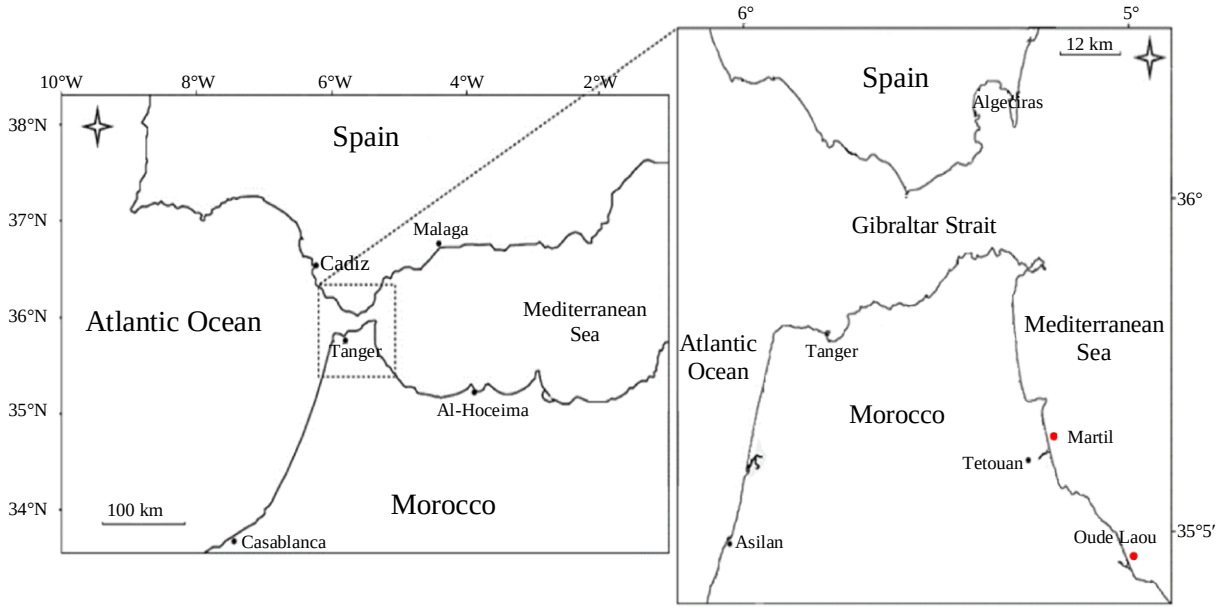

Fig. 1: Location of sampling sites

For chemical analysis, 150 individuals were washed with distilled water to avoid any type of contamination, then they were mixed, homogenized, lyophilized and stored until further analysis. For biochemical analysis, the digestive gland and gills of 50 individuals were excised and placed by 5 pools (ten individuals each of similar size for both organ). Then, they were immediately frozen in liquid nitrogen and stored at °C until biomarker analysis.

Analysis of PAH

The analytical procedure used to detect the aromatic contamination was as described by Azevedo *et al.* (2004) and recommended by the National Bureau of Standars (Wise *et al*., 1980) adjusted. In summary, hydrocarbons were extracted from 5 grams of lyophilized tissue using methanol and Soxhlet apparatus for 8 h (one cycle by hour). Prior to extraction, the C24D50 and Para D10 terphenyl standards were added to all samples. Saponification step by KOH/distilled water (0.7N) for 2 h was carried out in the aim of releasing hydrocarbons and removing saponifiable compounds from the lipid extract. Afterwards, liquid/liquid extraction was made with hexane (three times). Then the extract was rotary evaporated under reduced pressure until near dryness and then dried under a gentle stream of nitrogen.

The purification and fractionation phase were performed in column filled with 10 ml of neutral alumina/silica (v: v) (activated and deactivated). Aliphatic hydrocarbons were removed by elution with 20ml n-hexane and then PAHs were recovered by tow elution of n-hexane/dichloromethane: 20ml (9:1) and 30ml (7:3) respectively for low and heavy PAHs.

The qualitative analysis of PAHs was carried out using gas chromatography coupled with mass spectrometry (GC/SM) in a "Selected Ion Monitoring" mode (SIM). The GC/MS used was an HP6890 GC coupled to a HP5973 Mass Selective Detector, equipped with a DB-5MS fused-silica column (J&W, 30 m, 0.25 mm i.d.,0.25 µm film thickness). Used program of temperature was 60 to 100°C (25°C/min) and 100 to 310°C (2°C/min) with a level of 70 min at 310°C.

Biochemical Analysis

The biochemical study concerns some parameters such as proteins rate, activity of GST and AchE keys enzymes of detoxification and of the neurotoxicity respectively.

After thawing, the gills and digestive glands (pools of ten individuals) were homogenized in a 1:3 ratio (weight: volume) of phosphate buffer (pH 7). After centrifugation for 25 min at 9000 g, the supernatant obtained (called S9 fraction) was retained then divided into several aliquots and immediately kept at −80°C. All measures were performed in triplicate and all procedures were carried out at 4°C to prevent enzyme or tissue degradation.

Proteins content was determined by the method of Bradford (1976) using BSA as standard at 595nm.

Glutathion-S-Transferase (GST) activities were measured spectrophotometrically at 340 nm by following conjugation of the acceptor substrate 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (Habig *et al*., 1974). Acetylcholinesterase (AchE) activity was determined using the method of Ellman *et al.* (1961). The method measures the absorbance of 5-Thio-2-Nitrobenzoate (TNB) formed by the reaction of

thiocholine, a product of Acethylthiocholine (ATC) cleavage by AChE with 5,5-Dithio-bis-2 Nitrobenzoate (DTNB). The results of this activities were expressed in nanomole per min per mg protein.

Statistical Analysis

The results of various parameters were represented by values of average ± standard deviation of the mean (mean ± SD). All settings have been tested using analysis of variance (ANOVA) in order to analyze factors effects. Significant differences had been established at the p level p<0,05 according to the post-hoc of the Tukey HSD test. A Principal Component Analysis (PCA) was made to distinguish between these variables. These principal components analysis integrate and compare the results of all studied parameters in a graphical format, which facilitates their interpretation. Statistical analysis was performed using the STATISTICA software program (v. 6.1.478.0 Statsoft).

Results

Chemical Analysis

Polycyclic aromatic hydrocarbons were analyzed in cockles (*A.Tuberculatae*) collected from Martil and Oued Laou stations. The results are shown in Table 1. It shows total PAHs (ΣPAH) concentrations ranging from 12µg/g (dw) and 11.65 µg/g respectively in Martil and Oued Laou sites. Results of PAHs displayed a large range of composition ranging from di-aromatic compounds (Naphthalene) to five-aromatic compounds (Benzo-a-pyrene). PAHs composition showed two different profiles for Oued Laou and Martil stations. In this latter, 12 compounds of 16 PAHs described by EPA were found: Naphthalene (N), Acenaphthylene (Acyl), Acenaphthene(Ac), Fluorene (F), Phenanthrene (P), Anthracene (A), Fluoranthene (Fluo), Pyrene (Pyr), Chrysene (Chry), Benzo-b-Fluoranthene (BbF), Benzo-a-pyrene (BaP) and Perylene (Per). Furthermore, five substituted compounds were detected: 1-Methylnaphtalene (1-MN), 1Ethylnaphtalene (1-EN), 1-Methylphenantrene (1-MP), 2Methylphenantrene (2-MP) and 3,6-Dimethylphenantrene (3,6-DiMP).

Those compounds were also detected in Oued Laou site excepted for 5 compounds, namely 1-MN, 1-EN, 3.6-DiMP, BaP and Per. In addition of those compounds Benzo-a-Anthracéne (BaA) was detected in this site.

On another hand, specific ratios of PAHs compounds are largely used in the fingerprinting of origins; by means of several ratios such as P/A, Fluo/Pyr, BaA/Chry, A/(A + P), Fluo/(Fluo + Pyr), BaA/(BaA + Chry) and LMP/HPM. They allow a clear distinction between pyrolytic and petrogenic origins of PAHs (Budzinski *et al*., 1997; Zeng and Vista, 1997; Wang *et al*. 1999; Yunker *et al*., 2002; Ross and Oros, 2004; De Luca *et al*., 2005) (Table 2).

Table 1: Polycyclic Aromatic Hydrocarbons (PAHs) Concentrations in organisms in µg/g (dw)

	Sampling site	
Compounds	Oued laou	Martil
∑PAH	10.12	11.657
N	0.218	1.711
1-MN	-	0.077
1-EN	-	0.230
Acyl	0.022	0.010
Ac	0.033	0.012
F	0.033	0.094
P	0.116	0.233
1-MP	0.023	0.053
2-MP	0.019	0.144
3.6-DiMP	-	0.007
A	0.019	0.017
Fluo	0.041	0.042
Pyr	0.081	0.085
BaA	0.167	-
Chry	0.020	0.021
BbF	9.388	4.274
BaP	-	4.230
Per	-	0.462
LMW = ∑ 2-3 cycles	0.450	2.100
HMW = ∑ 4-6 cycles	9.640	8.600

Table 2: Values of diagnostical PAHs ratios in organisms registered in both sites Martil and Oued laou

	Martil site		Oued laou site		
Ratios	Values	origins	Values	origins	Reference
P/A	13,37 (>10)	petrogenc	6,1(between 4 and10)	pyrolytic	Budzinski *et al*. (1997)
Fluo/Pyr	0,49 (<1)	petrogenic	5,06(>1)	pyrolytic	Budzinski *et al*. (1997)
BaA/Chry	-	-	8,33(>1)	petrogenic	Zeng and Vista (1997)
A/(A+P)	0,068 (<0,1)	petrogenic	0,14(>0,1)	Pyrolytic	Yunker *et al*. (2002)
Fluo/(Fluo+Pyr)	0,33 (<0,4)	petrogenic	0,33(0,4)	petrogenic	Wang *et al*.(1977)
BaA/(BaA +Chry)	-	-	0,89(>0,2)	pyrolytic	Oross et Ross (2004)
LMW/HMW	0,2(<1)	pyrolytic	0,04(<1)	pyrolytic	De *luca et al*. (2005)

The calculated ratios showed both origins, petrogenic and pyrolytic ones. The first one was more detected in Martil site and the second one was more noted in Oued Laou Site.

Biochemical Parameters

Results related to enzymatic response are presented in Table 3. It clearly shows significant response of both activities GST and AchE traducing respectively induction and inhibition. Those responses were more marked in Martil than Oued Loau ($p<0.05$). The induction of GST activity and the inhibition were higher in gills ((56.62 and 17.51 nmol/min/mg at Martil and Oued laou respectively) than digestive glands (31.91 and 8,20 nmol/min/mg at Martil and Oued laou respectively). Similarly, the inhibition of AchE was higher in gills (1.40 and 8.56 nmol/min/mg at Martil and Oued laou respectively) than digestive glands (0.37 and 3.54 nmol/min/mg at Martil and Oued laou respectively).

Relationship Between Total PAH Concentrations and Biomarkers

The relationship between PAHs accumulated in cockles soft tissues and the biochemical parameters either in gills or in digestive glands were assessed by Principal Component Analysis (PCA) variables studied by PCA analysis correspond to averages of total PAHs concentrations, averages concentration of different PAHs compounds detected (N, Acyl, Ac, F, P, A, Fluo, Pyr, Chry, BbF, BaP, Per, 1-MN, 1-EN, 1-MP, 2-MP and 3,6-DiMP) and averages biomarkers activities ; GST in gills (GST (G)), GST in digestive glands (GST (Dg)), AchE in gills (AchE (G)) and AhcE in digestive glands (AchE (Dg))(Fig. 2).

Two PCA were realized. The first one had concerned both activities in both organs and total PAHs explaining 90.4% of total variance (Fig. 2A) while the second one replaced total PAHs with all aromatic compounds detected explaining 92.56% of total variance (Fig. 2B).

PCA represented in Fig. 2A shows the presence of a clear positive correlation between GST activity in both organs and total PAHs in one hand and a distinct negative correlation with AchE activity in another hand.

Correlation was well represented in the second analysis (Fig. 2B) and (Table 4). Indeed, three correlations were observed: (1) between GST (Dg), N and 1-MN ; (2) between GST (G), Pyr, F and 2-MP and (3) between AchE (Dg), AchE (B), Ac, Bap and BbF.

Table 3: Biomarker activity (nmol/min/mg) in the digestive glands and gills of *A. Tuberculatae* sampled from two sites Martil and Oued Laou

Biomarkers	Site Martil	Site Oued laou
GST(G)	56,62±7,95[a]	17,51±3,62[b]
GST(Dg)	31,91±3,13[a]	8,20±2,25[b]
AchE (G)	1,40±0,29[a]	8,56±1,18[b]
Ache(Dg)	0,37±0,03[a]	3,54±0,94[b]

Results are expressed as mean of the hot season ± SE (n = 5). For each parameter, different letters indicate significant differences (Tukey test, $p<0.05$), among sites

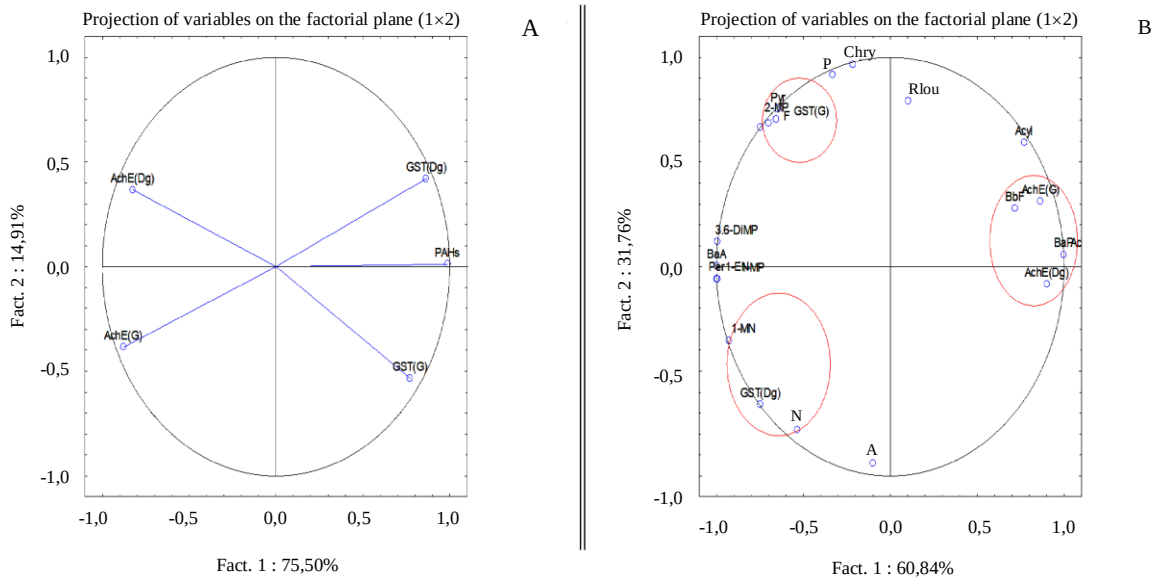

Fig. 2: Principal Component Analysis (PCA). (A) PCA of both activities in both organs and total PAHs. (B) PCA of all PAHs compounds detected and both activities

Table 4: Correlation matrix between chemical parameters and biochemical biomarkers analyzed in *Acanthocardia tuberculatae* collected from the two sites (Martil and Oued Laou) using Principal Component Analysis (PCA)

	GST(B)	GST(Gd)	AchE(B)	AchE(Gd)	HAPs	N	1-MN	1-EN	Acyl	Ac	F	Phe	A	1-MP	2-MP	3.6-DiMP	Fluo	Pyr	BaP	Chry	BbF	BaA	Per
GST(B)	1	0,056	-0,314	-0,772	0,667	-0,098	0,434	0,667	-0,177	-0,667	0,981	0,904	-0,509	0,638	0,884	0,774	0,585	0,939	-0,667	0,785	-0,185	0,706	0,667
GST(Gd)		1	-0,882	-0,586	0,780	0,890	0,912	0,780	-0,951	-0,780	0,118	-0,374	0,666	0,789	0,044	0,664	-0,645	-0,002	-0,780	-0,463	-0,767	0,743	0,780
AchE(B)			1	0,586	-0,863	-0,579	-0,851	-0,863	0,762	0,863	-0,433	0,085	-0,246	-0,913	-0,454	-0,828	0,576	-0,377	0,863	0,057	0,957	-0,854	-0,863
AchE(Gd)				1	-0,913	-0,553	-0,866	-0,913	0,742	0,913	-0,732	-0,467	-0,151	-0,853	-0,527	-0,896	-0,227	-0,584	0,913	-0,213	0,349	-0,911	-0,913
HAPs					1	0,593	0,948	1	-0,813	-1	0,709	0,286	0,167	0,991	0,596	0,982	-0,124	0,592	-1	0,153	-0,701	0,997	1
N						1	0,816	0,593	-0,950	-0,593	-0,117	-0,470	0,892	0,549	-0,291	0,431	-0,477	-0,287	-0,593	-0,685	-0,394	0,539	0,539
1-MN							1	0,948	-0,955	-0,948	0,456	0,014	0,470	0,926	0,313	0,872	-0,286	0,310	-0,948	-0,161	-0,665	0,926	0,948
1-EN								1	-0,813	-1	0,709	0,286	0,167	0,991	0,596	0,982	-0,124	0,592	-1	0,153	-0,701	0,997	1
Acyl									1	0,813	-0,182	0,240	-0,709	-0,781	-0,020	-0,689	0,411	-0,018	0,813	0,440	0,570	-0,774	-0,813
Ac										1	-0,705	-0,286	-0,167	-0,991	-0,596	-0,982	0,124	-0,592	1	-0,153	0,701	-0,997	-1
F											1	0,860	-0,547	0,701	0,925	0,820	0,414	0,979	-0,705	0,802	-0,342	0,748	0,705
P												1	-0,755	0,255	0,801	0,435	0,820	0,873	-0,286	0,926	0,157	0,338	0,286
A													1	0,123	-0,689	-0,021	-0,547	-0,688	-0,167	-0,932	-0,117	0,103	0,167
1-MP														1	0,631	0,981	-0,205	0,608	-0,991	0,170	-0,785	0,991	0,991
2-MPhe															1	0,737	0,315	0,988	-0,596	0,861	-0,451	0,647	0,596
3,6-DiMP																1	-0,021	0,732	-0,982	0,333	-0,689	0,992	0,982
Fluo																	1	0,439	0,124	0,650	0,683	-0,089	-0,097
Pyr																		1	-0,592	0,885	-0,342	0,643	0,592
BaP																			1	-0,153	0,701	-0,997	-1
Chry																				1	0,020	0,215	0,153
BbF																					1	-0,700	-0,701
BaA																						1	0,997
Per																							1

Discussion

The chemical analysis had shown an important accumulation of total PAHs. Their comparison to other studies seems to be difficult considering the lack of information on PAHs accumulation in this organism (*A. Tuberculatae*). Therefore, the comparison of these amounts was done referring to studies carried out in other bivalve species in the Mediterranean Sea and, thus, they appear to be relatively higher than what have been reported elsewhere; e.g., *Mytilus galloprovincialis, Ostrea edulis, Crassostrea gigas and Ruditapes decussata* at the Ebro Delta (NW Mediterranean) (Sole *et al*., 2000), in Mediterranean mussels (*M. galloprovincialis)* collected from north-western Adriatic coast (Trisciani *et al*., 2012) and in *Mytilus galloprovincialis* at Eastern Mediterranean coasts (Kasiotis *et al*., 2015).

On another hand, several studies have shown that certain molecules could be used in the fingerprinting of hydrocarbon inputs. Indeed, they provide deep information on hydrocarbons origins (Colombo *et al*., 1989; Khalili *et al*., 1995; Zeng and Vista, 1997; Wang *et al*., 1999).

Among this compounds Per, Fluo, Pyr, Chry and BaA are the most used. Per was often assigned to diagenetic processes and frequently used as a marker for natural and/or indigenous continental inputs while the other compounds usually reflect pyrolytic origin resulting from combustion phenomenon. However, the BaA presence even at low concentration could also indicates an oil origin (Zeng and Vista, 1997). Indeed, Fromme *et al*. (1988) highlight the impact of emissions linked to maritime traffic through this compound (BaA).

Effectively, the exam of chromatographic results had shown the presence of all those compounds at relatively similar concentrations, except for BaA in Martil and Per in Oued Laou (Table 1). This pattern of results indicates usually mixed origins; pyrolytic origin for both sites, petrogenic trace in Oued Laou and diagenetic processes in Martil.

Moreover, the exam of PAHs cycle's number was used to identify oil sources and provide additional diagnostic information. Indeed, petrogenic hydrocarbons are dominated by low molecular weight PAHs (LMW) while combustion processes generate mainly high molecular weight PAHs (HPM) (Colombo *et al*., 1989). In this case study, results showed the dominance of high molecular weight compounds in both sites, indicating pyrolytic origin (Table 1).

On another hand, the specific ratios of PAHs compounds used to fingerprint possible origins were calculated for all samples (Table 2). Inversely to advanced conclusions, the calculated ratios had shown important dominance of petrogenic origin in Martil site on one hand and on another hand mixed origin: petrogenic and pyrolytic in Oued Laou site.

In literature, although chemical analysis focus on qualitative and quantitative accumulation level, aromatic hydrocarbons impact on the studied organisms is not provided and stays deficient. Therefore, the use of biomarker approach seems to be required to differentiate between healthy and stressed organisms (Brooks *et al*., 2011).

Actually, the use of biomarkers for environmental monitoring comes to complete chemical analysis by providing an integrated evaluation of exposure to pollutants and its effects at different levels of biological organisms. This biological approach of disturbance impact on organisms and ecosystems is relatively recent (McCarthy and Shugart, 1990; Cairns and McCormick, 1992; Symposium, 1992; Peakall and Shugart, 1993; Fossi and Leonzio, 1993; Gestel and Brummelen, 1996; Lagadic *et al*., 1994; Lagadic, 1998).

In this regard, GST activity was suggested as an useful indicator of bivalves exposure to organic pollutants (Fitzpatrick and Sheehan, 1993). It plays an important role in the conjugation of electrophilic compounds with glutathione and these reactions are vitally important for the detoxification of xenobiotics (Kaaya *et al*., 1999) and are particularly involved in PAHs detoxification

(Hoarauetal, 2004; 2001). Several studies have reported significant induction of GST activity in mussels exposed to organic compounds (Moreira and Guilhermino, 2005; Rocher *et al*., 2006).

In this study, the analysis of GST activity variation at Martil compared to Oued Laou had shown significant increase of this activity ($p<0.05$) which confirms Martil site pollution by various contaminants including PAHs (Table 3). Indeed, Martil site is influenced by domestic, agricultural and industrial discharges that carry a wide variety of pollutants (Khannous *et al*., 2013). The GST activity increase suggests strong detoxifying activities in organisms as demonstrated by Bradai *et al*. (2007). The GST increase has also been reported in other bivalves such as *Mytilus edulis* and *Mytilusgalloprovincialis* exposed to PAHs effects (Narbonne *et al*., 2005 ; Trisciani *et al*., 2012). Besides, our data are consistent with results of pollution assessment studies in the Vigo and Pontevera estuaries in Spain, where significant positive correlations were found between GST induction and PAHs concentrations in gills of mussels (Vidal-Liñan, 2008).

The comparative analysis between the studied organs for the GST activity response revealed low levels in digestive glands compared to gills. Similar results were reported for *Mytilus sp* at the North Adriatic Sea (Italy), showing higher levels of GST in gills than digestive glands (Buratti *et al*., 2013; Vidal-Liñán and Bellas, 2013). The same response was found in clams *R. decussatus sp* at the Ria Formosa lagoon in Portugal (considered polluted), showing higher induction of GST activity at gills than digestive glands (Bebianno and Barreira, 2009).

Indeed, this GST induction much higher in gills than digestive glands was probably related to its role in the biotransformation of PAHs in organism's tissue. However, the GST response by an important induction (in gills) could be the result of an action as a peroxidas rather than as a Phase II enzyme (Sheehan *et al*., 2001; Varanasi *et al*., 1989).

Similarly, biochemical measurement of AChE, which is specific biomarker of neurotoxic effects, could be used to detect environmental stress and to reveal marine pollution. Many studies have reported AChE inhibition by various neurotoxic compounds such as organophosphate and carbamate pesticides (Bocquené and Galgani, 1998). Moreover, inhibition of this enzyme is also related to exposure to many other chemical groups, such as metals, hydrocarbons and detergents (Payne *et al*., 1996; Guilhermino *et al*., 1998; Elumalai *et al*., 2002; Kopecka *et al*., 2004).

This study results had highlighted an inhibition of AchE activity in both organs at Martil site compared to Oued laou site, which may reflect the effect of the presence of several neurotoxic compounds, including PAHs. In addition, as for GST activity, this response was more important in gills than digestive glands (Table 3).

Generally, the inhibition of AchE results from acetylcholine accumulation and subsequently on the functionality of cholinergic system, compromising both the filtration capacity as the ciliary motility (Cappello *et al*., 2013; 2015; D'Agata *et al*., 2014). Then this inhibition, which caused a reduction in the feed efficiency of bivalves (Yaqin *et al*., 2018), could be a physiological damage response in Martil cockles.

It appears clearly from these results that gills are the organ which manifests the most significant response to xenobiotics presence. Those results were consistent with other authors (Power and Sheehan, 1996) who indicated that GST activity is usually threefold higher in gills than in digestive gland.

Indeed, the location of gills in external mantle cavity of the organism makes them the principal entry point for the absorption of dissolved pollutants. They are the first organs exposed to pollutants and thus it is reasonable to expect that the level of oxidizing processes in this organ would be maximal (Soldatov *et al*., 2007). Therefore, they may represent a first line of defense especially against the more-soluble PAHs compounds accumulated through the gills (Cheung *et al*., 2001; Cossu *et al*., 2000).

In this study PCA analysis were performed to describe biomarker responses towards PAHs in cockles. The PCA analyses realized, showed a positive correlation between PAHs, GST (G) and GST (Dg) on one hand. On the other hand, it showed negative correlation between PAHs, AchE (G) and AchE (Dg) (Table 4), which is consistent with what has been reported in endobenthic organisms from estuaries contaminated with PAHs and PCB compounds (Durou *et al*., 2007; Cheung *et al*., 2001). These results were compatible with the GST role in the organism's defense exposed to chemical stress and the presence of neurotoxic compounds that are revealed from the inhibition of AchE's activity.

At the same time, results had shown positive correlation between total PAHs and GST activity, similar to what has been reported by other studies showing an increase of GST activity correlated with chemical concentrations (Durou *et al*., 2007). In our case the positive correlations were observed between the Nap, 1 – MpheNap and the GST activity in the gland in one hand, on the other hand, positive correlations were observed between Pyr, Fl, 2-MPhe and GST activity in gills which suggest that those three aromatic compounds could be the reason of oxydative stress in gills previoselly advanced.

According to the results of correlation, inhibition of AchE in both organs was probably due to the presence of Bap and BbF. In fact, persistent inhibition of acetylcholinesterase enzyme in mussels *M. galloprovincialis*has had been reported after 48 h of exposure to sublethal doses of Bap (Banni *et al*., 2010). These findings may explain the inhibition of AchE at Martil site, where the BaP was considerably present (Table 1).

Conclusion

The joint use of chemical analysis and biological responses appears to be necessary to detected impact of chemical products introduced into marine environment and consequently into marine organisms. This research was based first of all, on the assessment of PAHs levels in *Acanthocardia tuberculatae* sampled from two sites at Mediterranean West Moroccan coasts, then it focused in the study of enzymatic response (GST and AchE) to the presence of PAHs in two organs (gills and digestive glands).

Results of chemical analysis found showed mixed origins petrogenic and pyrolytic ones. Petrogenic origin was more dominant at Martil relatively to Oued Laou. This could be explained by important anthropogenic inputs in Martil corresponding to industrial and domestic discharges as well as shipping activity. On another hand, pyrolytic origin was more dominant at Oued Laou, due to combustion process.

Simultaneously, biochemical analysis had confirmed the stress of this sentinel species traduced by their enzymatic activity responses. This stress had been demonstrated by GST induction and AchE inhibition, more marked at Martil site. This impact could be due to the presence of certain polycyclic aromatic hydrocarbon compounds (N, 1-MN, Pyr, Fl, 2-MP, BaP) as suggested by the PCA results.

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Author Contributions

Several authors had contributed to manuscript elaboration.

Ikram Chbani: Had realized the sampling campaigns, laboratory analysis and had analyzed and interpreted the results in addition to the writing of the paper.

Noureddine Bouayad: Provided laboratory supervision for biomarkers analysis.

Saida Bouzid: Had participated to sampling campaigns, had provided laboratory supervision for PAH analysis and had contributed to the writing of the paper.

Hassan Er-Raioui: Assured the supervision of sampling campaigns, laboratory supervision and manuscript revision.

Ethics

All of the other authors have read and approved the manuscript and there are no ethical issues associated with this research.

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