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Research Paper

Chronic Effects of Petroleum Hydrocarbons in Tunis-Navigation Channel On Phase I and II Biotransformation Enzymes in Bivalve Species

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Abstract: *In the present study, the contamination state by hydrocarbons in Tunis-navigation channel was assessed and biotransformation biomarker responses in the clam Ruditapes decussatus were monitored. Sediments and clams R. decussatus samples were collected from the navigation channel of Tunis during four seasons. Total hydrocarbons (TH) were extracted from clam tissues and sediments and fractionated into non-aromatic hydrocarbons (NAH) and aromatic hydrocarbons (AH). In parallel to chemical analysis, benzo[a]pyrene hydroxylase (BPH) and glutathione S-transferase (GST) activities were measured in the digestive gland of the animal during four seasons. The results showed that the NAH and AH levels in the Tunis-navigation channel are higher than those recorded in the reference site (Louza). Moreover, hydrocarbon levels vary between seasons and reach their maximum in summer and spring. For biochemical analysis, we recorded a significant increase in BPH and GST activities compared with control clams. The biomarker responses (BPH and GST) fluctuate over time. In conclusion, results confirm a state of chronic pollution by petroleum hydrocarbons in the navigation channel affecting the biotic community and can have dangerous effect on human health.*

Keywords: petroleum hydrocarbons, sediments, clam, biotransformation enzymes, seasons.

Introduction

The marine environment is both witness and actor in the planet's history, and its chemical composition summarizes and integrates all of the complexities of its evolutionary processes. Marine pollution has, therefore, become a serious challenge, generating interest among several groups on the evaluation and the investigation of this pollution, and particularly petroleum pollution[1,2,3]. Contamination of the Mediterranean Sea by petroleum hydrocarbons is known to be caused by marine operations, run-offs by refineries and industry, and tanker accidents. The Mediterranean Sea is considered to be a representative model of the world's ocean. Being semi-enclosed, it also records various signals of high anthropic pressures from surrounding countries, as well as industrialized North European countries[4]. Hydrocarbon accumulations in marine sediment may be of biogenic or anthropogenic origin[5]. It has been recognized[6] that both petroleum and biogenic hydrocarbons have different chemical characteristics which are easy to distinguish; making the distinction between hydrocarbons and other sources is an important part of any objective petroleum contamination study.

Over the years, some marine bivalves have been widely and successfully used as bio-indicators for pollution monitoring in many coastal environments[7,8]. Filter-feeding invertebrates like mussels and clams live abundantly in the intertidal zone and tolerate a wide range of environmental conditions[9]. Many studies have demonstrated that several bivalve species have a great ability to accumulate organic pollutants and heavy metals and, thus, can be used as sensitive *in situ* indicators for pollution assessment[10,11,12]. It is always very difficult from only contamination body burden data to obtain information about their significance

upon animal health. Therefore, techniques for measuring biological effects are critical for any pollution monitoring program[13,14].

Biomarkers such as benzo[a]pyrene hydroxylase (BPH) and glutathione S-transferases (GSTs), which are involved in the metabolism of xenobiotic organic compounds, have often been assayed to indicate metabolisation of planar hydrophobic contaminants. BPH activity indicates CYP1A (a terminal component of the MFO system) activity in fish and molluscs[15]. The enzymatic activities of glutathione S-transferases (GST), a family of multi-functional enzymes involved in phase II of biotransformation are related to cellular antioxidant defenses due to the conjugation of electrophilic xenobiotics and oxidized components with glutathione (GSH)[16].

The main objective of this study is to investigate the accumulation hydrocarbons in the marine clam *Ruditapes decussatus* and sediments of the Tunis-navigation channel (Tunisia) and evaluate the contamination impact on detoxification enzyme activities during four seasons.

Material and Methods

Sampling

Located in the northern Tunisia, the Tunis-navigation channel separates the Tunis lagoon into two parts, north and south lagoon, and connects Rades and Goulette harbours to the sea. Surface sediment and *R. decussatus* samples were seasonally collected from the navigation channel (36°48'32.0"N 10°16'58.0"E), during one year. Control clams *R. decussatus* and sediments were collected from the Louza site (35°01'11.1"N 11°00'24.6"E), which considered as a reference site in monitoring programs along the Tunisian coasts[13] (Figure 1).

Samples of surface sediments were collected from the Tunis-navigation channel and Louza sites during four seasons. In the laboratory, sediment samples were manually homogenized, lyophilized and stored until analysis. Clams *R. decussatus* were collected seasonally from the same sampling sites. In the laboratory, digestive gland were rapidly dissected out and stored at -80 °C until biomarker analysis. The whole soft tissues of clams were extracted, freeze-dried, and homogenized for subsequent chemical analysis of hydrocarbons.

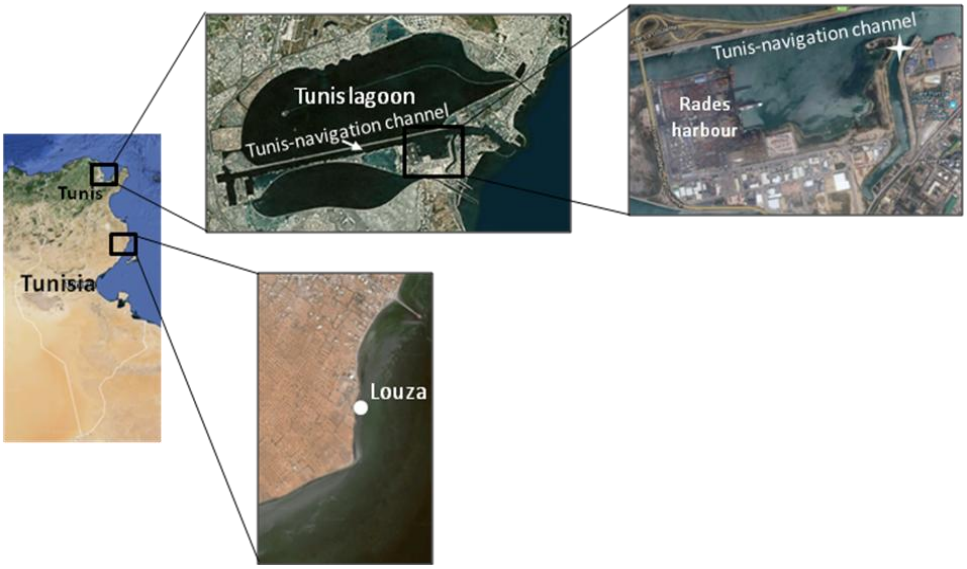

Figure 1: Map of the study area and location of the sampling sites in Tunis-navigation channel and Louza

Chemical analysis

Hydrocarbons were extracted according to the method described in Er-Raioui et al.[17] with some modifications. The analytical procedure used for Total Hydrocarbon (THC) extraction from the sediments and clam tissues consisted of the extraction of 50 g (wet weight) from the sediment samples and 5 g for the lyophilized bivalve tissues in a soxhlet for 8 hours. Saponification process is necessary for tissue samples to allow hydrocarbons liberation from lipids, 20 mL of 0.7 M KOH and 30 mL of distilled water were added to the flasks and the extraction prolonged for another 2 hours in order to saponify the lipids. At the end, the content of the extraction flasks were extracted three times with hexane to recover the non-saponified lipids. Following solvent evaporation, each extract was fractionated into aliphatic and aromatic hydrocarbons by adsorption liquid chromatography using a column of silica-gel, and gradient solvents as eluent: n-hexane and n-hexane/chloroform (2:1 v/v) for non-aromatic hydrocarbon (NAH) and aromatic hydrocarbon (AH) fractions, respectively. The total petroleum hydrocarbon (TPH) represents the sum of the NAH and AH fractions. The different extracts were weighed with a microbalance for THC, NAH and AH determination. Results were expressed as relative hydrocarbons concentration in navigation channel compared to hydrocarbons

concentration in reference site: Relative hydrocarbons level = [hydrocarbons]nc / [hydrocarbons]rs.

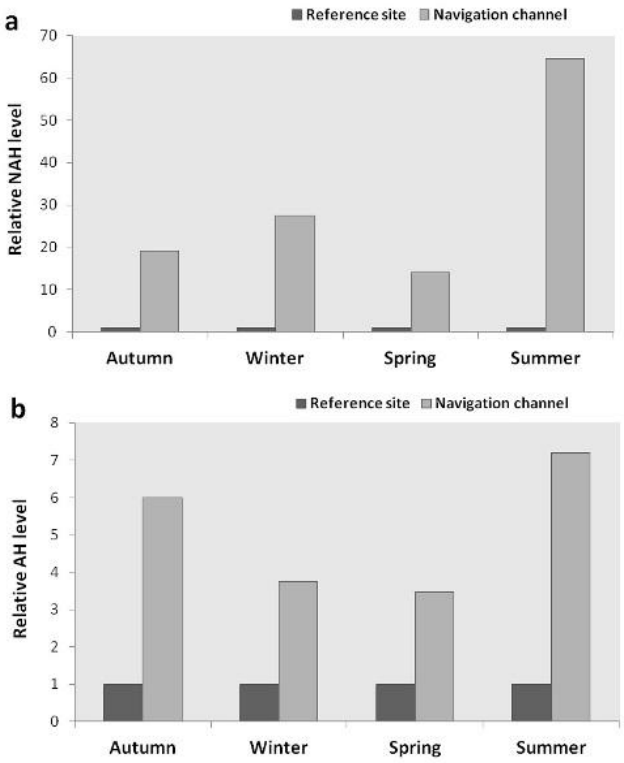

Figure 2: Seasonal variation of (a) non aromatic (NAH) and (b) aromatic (AH) hydrocarbons contents in the surface sediments sampled from Tunis-navigation channel and reference site. Hydrocarbons levels were performed respect to the reference site

Biochemical analysis

Before biochemical analysis, digestive glands were homogenized in phosphate buffer (0.1 M, pH 7.5). The homogenate obtained was centrifuged at 100 000 g for microsomal fraction or at 9000 g for cytosolic fractions (S9). The quantities of proteins present in microsomal fraction and in S9 fraction were determined according to the Bradford[18] method using Coomassie blue reagent and bovine serum albumin as standard protein.

BPH activity was assessed in digestive gland microsomes by the adapted fluorometric method of Michel et al.[19] to a microplate reader. Following incubation with B[a]P, there action was stopped by the addition of 10% TritonX-100. Fluorescence of the sample was obtained by difference in fluorescence between the respective emission/excitation wavelengths of 492/430 and 510/430 nm. A known amount of 3-OHB[a]P was used as internal standard in all samples to control for quenching. Results were expressed in pmoles per min and per mg proteins.

GST activity was measured in digestive gland cytosol by the method of Habig et al.[20] using 10 mg of cytosolic protein, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma-Aldrich, Saint Louis, MO, USA) as substrate and 4 mM glutathione reduced form (GSH), in 100 mM sodium phosphate buffer, pH 7.5. GST activity was determined by kinetic measurement at 25°C a spectro UV-VIS double beam PC scanning spectrophotometer UVD-2960 (λ = 340 nm). Results were expressed as nmoles GSH-CDNB produced per min and per mg proteins.

Statistical analysis

Biochemical data was expressed as mean ± standard deviation (SD). Data statistical analysis was performed using one-way analysis of variance (ANOVA) and Duncan's test for multiple range comparison, $p < 0.05$ was considered as significant. Statistical analysis was carried out with SigmaStat software 3.5 (Systat Software, Inc.).

Results and Discussion

Hydrocarbons levels in sediments and tissue of clams

The results of hydrocarbons contents in sediments during the four seasons are reported in Figure 2.

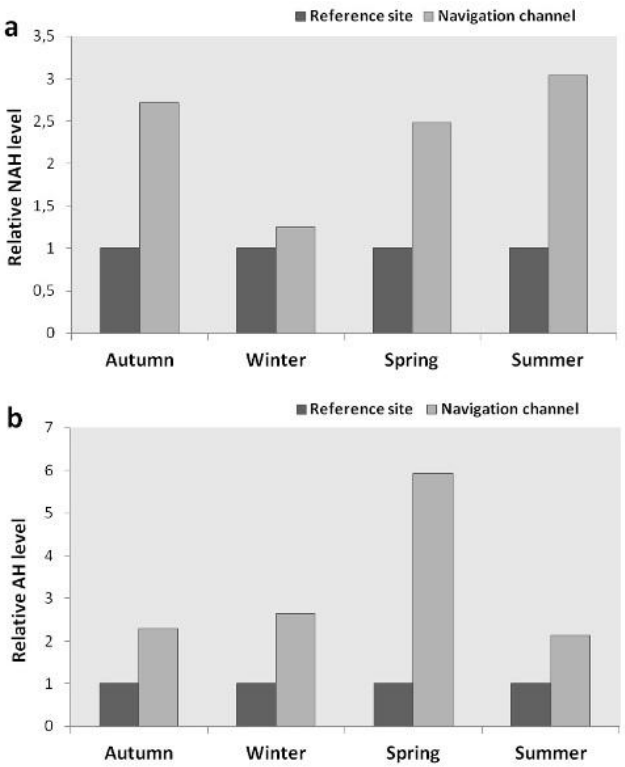

Figure 3: Seasonal variation of (a) non aromatic (NAH) and (b) aromatic (AH) hydrocarbons contents in the soft tissue of clam *Ruditapes decussatus* collected from Tunis-navigation channel and reference site. Hydrocarbons levels were performed respect to the reference site

The results show an increase of NAH and AH contents in sediment samples from navigation channel compared to the reference site, with a seasonal variation. Data show a maximum value in summer for NAH with

64.6-fold increase compared to reference site. A significant increase of sediment AH level in navigation channel was recorded during summer and autumn with 7.2- and 6-fold increases, respectively, compared to reference site.

Similarly, clams from contaminated site show higher petroleum hydrocarbon levels (NAH and AH) than control clams (Figure 3). The NAH level increase in clams from navigation channel is very pronounced during summer, autumn and spring with 3-, 2.7-, and 2.5-fold increases, respectively, compared with control clams. While, the maximum value of AH concentration was observed in spring with 5.9-fold increase compared with control clams.

Biotransformation enzymes

Figure 4 shows the seasonal variation of BPH activity in digestive gland of *R. decussates* sampled from the Tunis-navigation channel and the reference site. Clams from the contaminated site presented increased levels of BPH than those collected at the reference site during the four seasons. The maximum BPH activity was reached during spring and summer with values equal to 159.7 and 162.9 pmol/min/mg proteins, respectively.

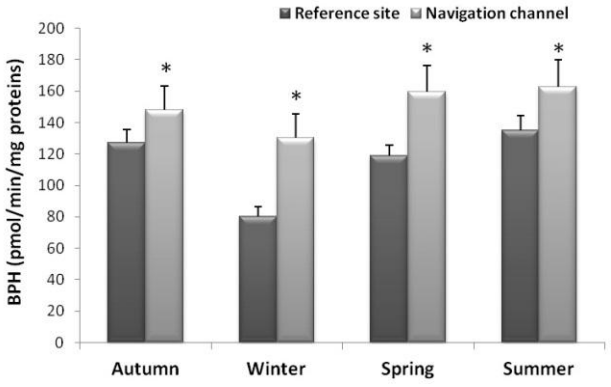

Figure 4: Response of benzo[a]pyrene hydroxylase activity in the digestive gland of clam *Ruditapes decussatus* collected from Tunis-navigation channel and reference site. Results are expressed as mean ± SD (*n* = 10). * indicates *p* < 0.05 compared with reference site (significantly different by ANOVA, multiple comparison, and Duncan's test across sites)

The results relative to the seasonal variation of GST activity in clams sampled from the Tunis-navigation channel and the reference site are reported in Figure 5.

Digestive gland GST activity in contaminated clams showed an increasing trend response during all seasons when compared to control clams with a seasonal fluctuation. The maximum GST activities were observed during autumn and spring with values equal to 502.5 and 475.2 nmol/min/mg proteins, respectively.

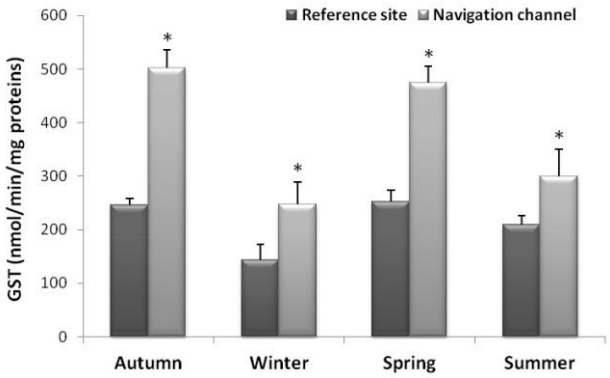

Figure 5: Response of glutathione S-transferase activity in the digestive gland of clam *Ruditapes decussatus* collected from Tunis-navigation channel and reference site. Results are expressed as mean ± SD (*n* = 10). * indicates *p* < 0.05 compared with reference site (significantly different by ANOVA, multiple comparison, and Duncan's test across sites)

Discussion

To assess the contamination state of the marine environment of Tunis-navigation channel, we used a quantitative study for determining the levels of the different hydrocarbon fractions in marine sediments and clam tissues. In the present paper, we reported enrichment in NAH and AH in both matrixes (sediments and clams) collected from navigation channel compared to the reference site (Louza). Moreover, the levels of hydrocarbons present a seasonal variation and the maximum values are generally reached during summer. This study demonstrates that *R. decussatus* and sediments were exposed to a recent small spill or to continuous exposure to low levels of petroleum as the source of these pollutant hydrocarbons. In the study area, the main petroleum hydrocarbon source is thought to be shipping traffic; also the presence of Rades harbour in side to the study area can contribute to environmental contamination by oil hydrocarbons. During the summer season, evaporation along with a high concentration of hydrocarbons in the seawater following maritime traffic lead to the accumulation of oil hydrocarbons in the clam tissues[21].

During winter, the mollusc tissues decontaminate, thanks to their immersion in water that is much less polluted following the contribution of rain water and the ebb and flow of seawater currents. Accordingly, bivalve not exposed to the pollution sources during a few weeks gradually decontaminate[22]. Our results are in accordance with those found by Khedir-Ghenim et al.[21], which showed a contamination of clams and seawater in the navigation channel of Tunis by petroleum hydrocarbons.

The chemical analysis allowed us to confirm contamination of the Tunis-navigation channel by

petroleum hydrocarbons (aromatic and non aromatic). Thereafter, we used the biomarkers in the clam *R. decussatus* to assess the biological impact of the environmental contamination on this species. The adaptability of bivalves exposed to hydrocarbons can be estimated from changes in enzyme activities such as benzo[a]pyrene hydroxylase (BPH), used as biomarkers of detoxification. In the present paper, we reported a significant increase of BPH activity in the navigation channel during all season compared to control values. This suggests an increase of the phase I biotransformation process and hence, a higher production of phase I metabolites. The comparative study conducted by Suteau et al.[23] showed a 50% increase in BPH activity in mussels *Mytilus galloprovincialis* collected from contaminated sites by PAH in the Mediterranean coasts of France compared with mussels collected from reference sites. The seasonal study showed a variation of BPH activity. This seasonal variation arises from a complex interaction between exogenous factors such as food availability, temperature, growth and contaminants and endogenous factors such as reproductive activities[13].

GST activity is part of the phase II of the metabolism process, involved in the metabolism of organic xenobiotics, they have always been measured to determine the metabolism of planar hydrophobic contaminants[24]. Our data showed a significant increase of GST activity in our contaminated sampling site compared to the control values with a seasonal variation. This activity increase confirms pollution of the navigation channel of Tunis by organic contaminants. Hoarau et al.[25] determined an increase in GST activity in clam *Ruditapes decussatus* exposed to B[a]P concentrations. Similarly, Bebianno and Barreira[24], showed an induction of GST activity in the digestive gland and gills of clams *Ruditapes decussatus* transplanted in a polluted site (Ria Formosa lagoon, Portugal). Power and Sheehan[26] have revealed that the GST are much less sensitive to seasonal variations than indicators of oxidative stress and phase I metabolism components.

Conclusion

The present study demonstrated a contamination state of Tunis-navigation channel by petroleum hydrocarbons due to the presence of different sources such as harbour, industrial activities and shipping traffic. The presence of hydrocarbons in clam tissues confirmed the bioavailability of hydrocarbons for benthic organisms. Furthermore, this study shows a seasonal variation in the level of petroleum contamination and enzymatic activity in *R. decussatus* which can be attributed to the complex interaction between the abiotic environmental conditions and the biological parameters of the organism. These kinds of studies provide an important means for identifying the potential effects of contaminant impacts, particularly at areas that are experiencing chronic exposures.

Acknowledgement

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